CORTISOL METABOLISM IN TYPE 2 DIABETES MELLITUS

DR ROBERT ANDREWS

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Recent evidence suggests that increased cortisol secretion, altered cortisol metabolism and/or increased tissue sensitivity to cortisol may link insulin resistance, hypertension and obesity. Whether these changes are important in type 2 diabetes mellitus (DM) at the time of starting this thesis was unknown. The work encompassed in this thesis aimed to answer this question and for the first time examined whether inhibition of local tissue metabolism of cortisol, by blocking 11 ß-hydroxysteroid dehydrogenases type 1 and 2 (11ß-HSD 1 and 2), could be a novel therapeutic target for enhancing insulin sensitivity in patients with DM.

In the introduction to this thesis, I have described the mechanisms that are implicated in the pathogenesis of DM and highlighted the potential relevance of cortisol to these. I have also discussed factors that modulate the effect of cortisol on these mechanisms and focused on a novel therapeutic strategy to manipulate cortisol action. Finally I have described evidence from human studies that demonstrate the importance of cortisol in the pathogenesis of many of the abnormalities commonly associated with DM.

In chapter 2, I describe my first study, which determined whether individuals with DM or impaired glucose tolerance (IGT) exhibit abnormalities in cortisol activity. An integrated assessment of cortisol secretion, metabolism and action was carried out in 25 un-medicated lean male patients with hyperglycaemia (20 DM and 5 IGT) and 25 healthy controls carefully matched for body mass index, age and blood pressure. This study demonstrated that patients do exhibit abnormalities in cortisol activity with: 1) normal cortisol secretion and circulating levels in the face of enhanced negative feedback sensitivity (as measured by 0.25mg of dexamethasone); 2) enhanced *in vivo* peripheral tissue sensitivity to glucocorticoids (as measured by dermal blanching to beclomethasone); 3) impaired hepatic 11ß-HSD1 activity and normal adipose 11ß-HSD1 activity, suggesting tissue-specific alterations in 11ß-HSD1 activity; and 4) increased relative excretion of A-ring reduced metabolites of cortisol.

Chapter 3 describes a study that examined whether altered tissue concentration of the glucocorticoid receptor (GR), of 11ß-HSD1 or of 11ß-HSD2 could explain the difference in dermal blanching seen between patients with hyperglycaemia and normal healthy controls. Unfortunately I was unable to measure tissue 11ß-HSD1 and 11ß-HSD2 but tissue concentrations of GR were found to be no different between patients with hyperglycaemia and normal healthy subjects.

In chapter 4, I describe a study, which assessed whether inhibition of local tissue metabolism of cortisol, by carbenoxolone (an inhibitor of both 11ß-HSD1 and 11ß-HSD2) improved insulin sensitivity. 6 patients with DM and 6 matched controls, participated in a double-blind cross-over comparison of carbenoxolone (100 mg 8 hrly orally for 7 d) and placebo. At the end of each phase glucose kinetics were measured in the fasting state from 07:00-07:30 h, during a 3 h euglycaemic hyperinsulinaemic clamp and during a 2 h euglycaemic hyperinsulinaemic clamp with a 4-fold increase in glucagon levels. Carbenoxolone reduced total cholesterol in healthy subjects but had no effect on cholesterol in patients with DM. Carbenoxolone did not affect insulin sensitivity, but it did reduce glucose production rate during hyperglucagonaemia in patients with DM

In conclusion I have demonstrated that abnormalities in cortisol activity are seen in patients with DM and that drugs specifically targeted at preventing cortisol regeneration in tissues may enhance insulin sensitivity and lead to novel developments in the treatment of DM.

E: DECLARATION OF ORGINALITY

The studies presented in this thesis were designed and carried out by myself, unless otherwise stated, under the supervision of Professor Brian Walker at the Endocrinology Unit, Department of Medical Sciences, Western General Hospital, Edinburgh between 1997 and 2003. Work from collaborations with other individuals is indicated in the text and assays not performed by myself are also included in the text.

F: THE AUTHOR

Dr Robert C Andrews graduated with MB ChB from the University of Birmingham in 1991 and obtained Membership of the Royal College of Physicians (MRCP UK) in 1995.

The work presented in his thesis was undertaken by the Author while he was a Research Fellow in the Endocrinology Unit, Department of Medical Sciences, Western General Hospital, Edinburgh, UK, under the supervision of Professor Brian Walker between 1997-2000.

This thesis is dedicated to my mother and fiancé who has been supportive through both the highs and the lows.

H: Abbrevations

α	**Alpha**
β	**Beta**
CBG	**Cortisol binding globulin**
GR	**Glucocorticoid receptor**
HPA axis	**Hypothalamic-pituitary-adrenal axis**
IGT	**Impaired glucose tolerance**
IRS-1	**Insulin receptor substrate 1**
IRS-2	**Insulin receptor substrate 2**
PEP-CK	**Phosphoenolpyruvate carboxykinase**
T2DM	**Type 2 diabetes mellitus**
11β-HSD1	**11 beta-hydroxysteroid dehydrogenase type 1**
11β-HSD2	**11 beta-hydroxysteroid dehydrogenase type 2**
HOMA	**Homeostatis Assessment Model**
IVGTT	**Intravenous glucose tolerance test**

Chapter 1
Introduction

General introduction and overview

Glucocorticoids are so named because it was recognised long ago that one of their main actions is to increase blood glucose concentrations (Dallman et al., 1993). These hormones (mainly cortisol in man; corticosterone in rodents) also play a key role in regulating salt and water metabolism, blood pressure and immune function. Part of the mechanism causing these effects depends on opposing the actions of insulin, i.e. inducing a state of insulin resistance. For this reason glucocorticoids were one of the first hormones that were implicated in the development of Diabetes Mellitus. Early studies, though, found that cortisol secretion was normal in these individuals steering research away from this field (Mortimore et al., 1956).

Further investigations into the pathogenesis of diabetes led researchers to realise that there were in fact 2 forms of diabetes. One in which the onset was early, acute, more severe and associated with insulin sensitivity (Type 1 diabetes) and the other which was late onset, was less severe and associated with obesity and insulin resistance (Type 2 diabetes) (Lawrence, 1951). These finding should have prompted scientists to re-examine the role of glucocorticoid function in a population made up purely of individuals with Type 2 Diabetes (T2DM), but it was not until the early 1990s that interest in glucocorticoids would remerge.

Gerald Reaven's proposal that the conditions making up the "metabolic syndrome": impaired glucose tolerance, obesity, essential hypertension and coronary artery disease could all be linked by a common cause (Reavan, 1988) was the catalyst. Realisation that individuals with Cushing's syndrome, an excess of cortisol, exhibited many of these abnormalities was the first step. Studies of groups with hypertension, obesity and coronary artery disease followed (Walker et al., 1991) (Andrew et al., 1998) (Walker, 1996), all of which confirmed that abnormalities in glucocorticoid function were present. Further research in this field has led many to believe that abnormalities in cortisol could be the link that explains why the diseases found in the metabolic syndrome tend to cluster together (Andrews & Walker, 1999) (Bjorntorp et al., 1999) (Phillips et al., 1998). Whether abnormalities in glucocorticoids are important in the pathogenesis of T2DM remains to be elucidated.

In this introduction I will describe the mechanisms that have been implicated in the pathogenesis of T2DM and highlight the potential relevance of glucocorticoids. I will then discuss factors that modulate the effect of glucocorticoids on these mechanisms and focus on a novel therapeutic strategy to manipulate glucocorticoid action, which may prove useful in treating subjects with T2DM and associated diseases. Finally I will describe evidence from human studies that demonstrates that glucocorticoids are important in the pathophysiology of many of the abnormalities commonly associated with T2DM.

Type 2 diabetes

Type 2 diabetes is the most common type of diabetes, accounting for 85% of cases worldwide. It is currently undergoing a worldwide epidemic its prevalence having tripled in the last 30 years. Recent surveys in Europe indicate a prevalence of almost 5%, in the middle-aged and older population (Amos et al., 1997). The disease typically affects adults over the age of 40y and is strongly associated with obesity (over 80% of patients with T2DM are obese), inactivity, family history of diabetes and ethnic background. In the last 10 years there has been a shift towards a younger age onset and an emerging epidemic of the disease in children, adolescents and young adults, most notably in the USA and Japan (Nolan, 2002).

Diabetes related complications are a major cause of disability and suffering, and incur costs of 5-10% of gross national health budgets. Diabetes remains the principal cause of renal failure, limb amputations and blindness, and is also a major risk factor for heart disease and stroke (Bagust et al., 2003).

Pathophysiology of Type 2 diabetes

T2DM is a chronic, progressive metabolic disorder characterised by defects in both insulin action and insulin secretion. Although debate continues about whether insulin resistance or impaired insulin secretion is the primary initiating event on the road to the development of diabetes, both are clearly implicated in the development of frank diabetes. In patients with established T2DM, basal hepatic glucose output is increased (hepatic insulin resistance), insulin stimulated muscle glucose uptake is markedly reduced (peripheral insulin resistance) and, despite high concentrations of circulating insulin, the beta-cell (β cells) response to hyperglycaemia is inadequate (impaired insulin secretion) (Kahn, 1994).

Insulin Secretion

Insulin is composed of 2 peptide chains, A and B, linked by a disulphide bond. Synthesised as a larger single polypeptide, pre-proinsulin, by the beta cells in the Islets of Langerhans it is soon cleaved to form proinsulin. This is then packaged into

vesicles where it remains until required. When stimulation occurs these vesicles move to the cell surface, proinsulin is cleaved and insulin and c-peptide released.

In general insulin secretion is stimulated under circumstances of fuel excess and is inhibited under circumstances of fuel deficiency. The major stimulation to insulin secretion is a rise in blood glucose concentration, although certain amino acids, such as arginine, gastrointestinal hormones, glucagon, growth hormone and activation of the parasympathetic nervous system can also stimulate insulin secretion.

Insulin secretion is biphasic. The first phase insulin response is a rapid increase in plasma insulin concentration to a peak concentration at 2-4 minutes. Insulin concentration then decreases to a nadir at 10-15 minutes followed by a gradual progressive increase to a steady state at 2-3 hours, the second phase insulin release (Gerich, 2002). The first phase insulin response is believed to be important in suppressing hepatic glucose output and the second phase response in stimulating glucose uptake in muscles and adipose tissues (Del Prato et al., 2002).

Measurement

Insulin secretion "*in vivo*" can be measured by a variety of techniques. Oral glucose tolerance tests (OGTT) or mixed meals can be used to assess both phases of insulin secretion, with insulin measured every 5 minutes for 2 hours after ingestion. Unfortunately these tests do not provide a standard glucose stimulus as absorption can vary and this means that they lack accuracy, specificity and are difficult to reproduce (Ferrannini, 1998).

One-way around this is to provide an IV glucose load (IVGTT), thus standardising the glucose stimulus (Ferrannini & Pilo, 1979). Using these techniques an accurate measure of acute insulin release in response to a glucose load can be made. The insulin response to this IV glucose load tends not to be biphasic and only provides information about insulin secretion under maximal stimulation. How this relates to the oscillatory patterns of minute to minute insulin release is not known. Recent use of glucose clamps and deconvolution analysis has allowed dose response curves to

be constructed and oscillatory patterns of insulin secretion throughout the day to be examined. The finding that insulin secretion is affected by age, race, sex, physical fitness, BMI, and prevailing glucose and insulin concentrations (Clausen et al., 1996) (Kahn, 2003) has led many researchers to re-examine previous data and again question the role that insulin secretion plays in the pathogenesis of T2DM.

Evidence that insulin secretion is important in the development of diabetes

Relative or absolute insulin deficiency is one of the characteristics which defines diabetes (WHO Expert Committee on Diabetes Mellitus, 1980). No matter how insulin resistant an individual is, T2DM will only occur when the pancreas fails to secrete enough insulin. Early theories on the pathogenesis of diabetes suggested that in the prediabetic state when glucose tolerance is normal insulin resistance is already present, and that a compensatory increase in insulin secretion maintains normal glucose tolerance. Progression to impaired glucose tolerance (IGT) occurs because of worsening insulin resistance without an appropriate increase of insulin secretion. IGT deteriorates into overt diabetes when β-cells become exhausted after years of greater than normal insulin secretion. Improvements in techniques used to measure insulin secretion have challenged this view and some individuals have suggested that abnormalities in insulin secretion occur before the development of insulin resistance (Saad et al., 1998).

In individuals at risk of developing IGT and T2DM, abnormalities of insulin secretion have been described. First-degree relatives of patients with T2DM have a lower first phase insulin response to a glucose load than those without a family history (Bogardus & Tataranni, 2002). Offspring of diabetic pregnancies also have a lower first phase insulin response when compared to offspring of normal pregnancies (Bogardus & Tataranni, 2002). Studies of identical twins, discordant for T2DM, demonstrate impairment in β-cell function well before the onset of IGT or T2DM (Cerasi & Luft, 1967) (Barnett et al., 1981) (Pyke & Taylor, 1967). In the only twin study that simultaneously examined insulin sensitivity and insulin secretion, first phase insulin secretion was found to be reduced at a time when insulin sensitivity was normal (Vaag et al., 1995).

The importance of this impaired β-cell function in the pathogenesis of T2DM has also been demonstrated in recent cross sectional studies. In the San Antonio Heart Study a low incremental 30 minute insulin concentration (an indicator of first phase insulin response) was predictive of the progression of normal glucose tolerance to impaired glucose tolerance in a Mexican-American population (Haffner et al., 1996). A reduced insulin secretion response was also found to be predictive of the development of T2DM in a population made up of Pima Indians (Bogardus & Tataranni, 2002).

Because of this evidence many researchers believe that loss of first phase insulin is the first and most important factor in the development of T2DM. Further evidence for this comes from research which has looked at the effect of blocking the first phase insulin response. When somatostatin is used to block this response, glucose tolerance deteriorates (Del Prato et al., 2002).

In 1995 Zowalich proposed different mechanisms by which abnormalities in insulin secretion causes T2DM (Zawalich & Kelley, 1995). He proposed that the first abnormality seen in patients destined to get T2DM is enhanced β-cell responsiveness to glucose, a result of increases in vagal and fuel stimulation. This increase in β-cell responsiveness leads to chronic hyperinsulinaemia, which in turn is then responsible for changes in target tissue sensitivity to the glucose-regulatory effect of insulin (insulin resistance). Although somewhat speculative this theory does explain why hyperinsulinaemia is seen to predate T2DM (see below), why lowering insulin levels restores insulin sensitivity, and why diabetes flourishes in populations where over nutrition is apparently the major precipitating event in disease emergence (Zimmet, 1982)(Zimmet et al., 1984).

So whether it is loss of first phase insulin secretion or enhanced β-cell responsiveness to glucose there is a large body of evidence that suggests that abnormalities in insulin secretion are important in the pathogenesis of T2DM.

How do glucocorticoids affect insulin secretion?

Both animal and human studies have clearly demonstrated that high plasma concentrations of glucocorticoids can inhibit insulin secretion (Delaunay et al., 1997) (Ling et al., 1998) (Lambillotte et al., 1997). Animals chronically exposed to high concentrations of glucocorticoids show increased islet cell volume but a decreased insulin response to glucose (Ogawa et al., 1992). In healthy individuals the decrease in insulin secretion seen with dexamethasone is dose dependent, with higher doses causing greater suppression (Matsumoto et al., 1996).

The role that physiological concentrations of glucocorticoids play in determining insulin secretion is less clear. In 1995 Plat et al demonstrated that a small increase in plasma cortisol concentrations, induced by low doses of IV hydrocortisone or low doses of IV corticotrophin-releasing hormone (CRH), abruptly inhibited insulin secretion without changes in glucose concentrations (Plat et al., 1996). Further studies though are required to confirm or refute these findings.

The exact mechanism by which glucocorticoids decrease insulin secretion has yet to be proven. They could have a direct action on glucocorticoid receptors in the pancreas, somehow affecting the efficiency of calcium on the insulin secretory pathway (Lambillotte et al., 1997). Alternatively the rise in glucose or free fatty acids induced by cortisol could have a toxic effect on the pancreas resulting in less insulin secretion (Toschi et al., 2002) (Lupi et al., 2002).

The importance of the effect of glucocorticoids on insulin secretion is most markedly seen in individuals who are on high doses of glucocorticoids. All of these individuals show evidence of increased insulin resistance but only a few develop glucose intolerance and only a handful develop frank diabetes. All those who develop impaired glucose tolerance or diabetes show a decrease in insulin secretion, whereas those who maintain normal glucose tolerance retain normal insulin secretion (Wajngot et al., 1992).

Insulin resistance

Once insulin has been secreted, it circulates unbound to its target tissue. Here it binds to a cell-surface receptor made up of two alpha (α) and two beta (β) subunits. Binding induces a conformational change in the β subunit, which in turn activates tyrosine kinase, so called phosphorylation of the receptor (figure 1.1). Once activated, tyrosine kinase, phosphorylates intracellular proteins; such as insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2). It is through these second messengers that insulin has its effects. In broad terms insulin actions can be divided into the regulation of long-term growth and short-term metabolism, both of which are mediated by different intracellular second messenger signalling pathways (Baynes et al., 1997).

Definition

The term 'insulin resistance' was first coined by Himsworth in 1936 (Himsworth, 1936) and has subsequently been defined as "a state in which a given concentration of insulin produces a less than normal biological response" (Kahn, 1994). In most circumstances insulin resistance is used to refer to the acute regulation of carbohydrate metabolism by insulin, but it could just as easily be used to describe the effect of insulin on growth.

Measurement

Insulin action *in vivo* can be measured by a variety of techniques. Some of these use mathematical models (Homeostasis Assessment Model (HOMA) and intravenous glucose tolerance test (IVGTT)), others measure the rate of decline in glucose to a given insulin load (insulin tolerance test (ITT)) and others measure the amount of glucose needed to be infused to maintain glucose concentration at predetermined levels, whilst infusing insulin at a constant rate (euglycaemic clamps and hyperglycaemic clamps).

HOMA is a mathematical model which allows values for insulin sensitivity and β cell function to be obtained if simultaneous fasting plasma glucose and fasting

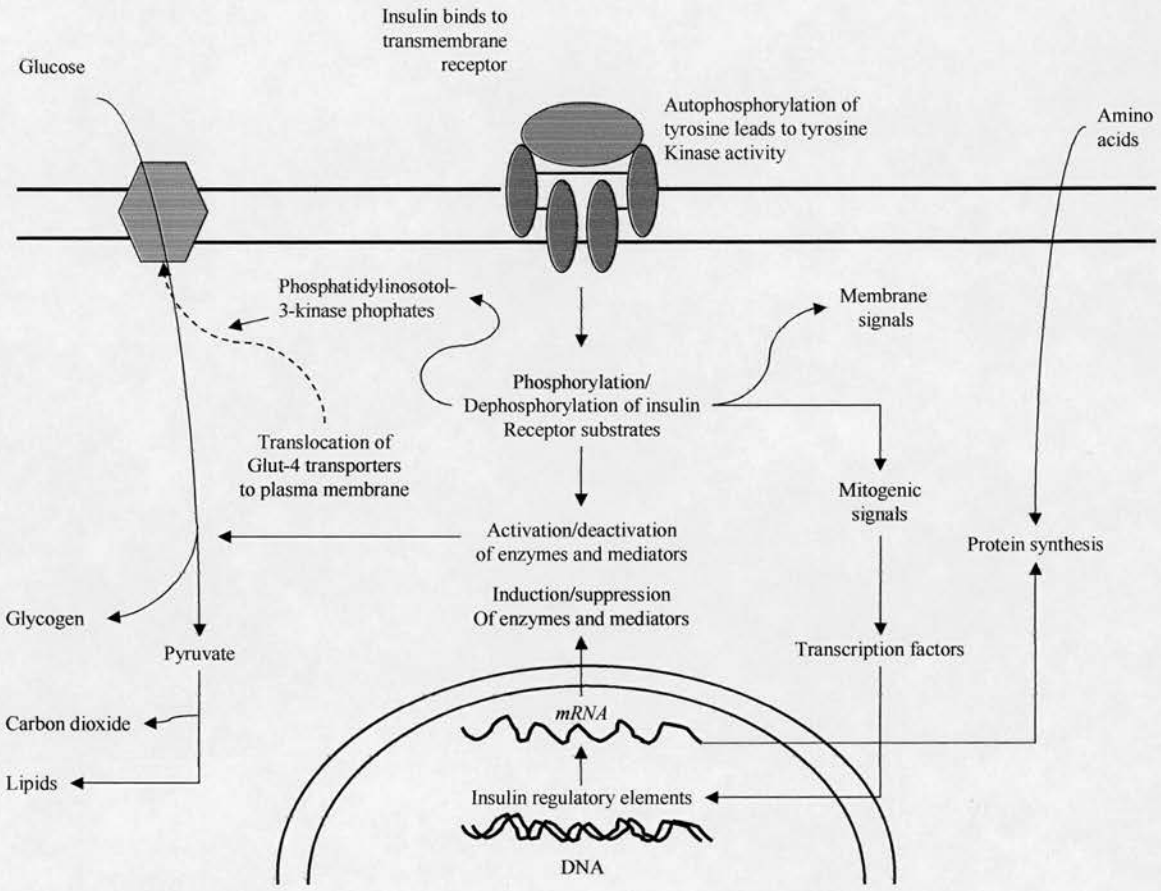


Figure 1.1. Mechanism of insulin action
A schematic for an archetypal insulin-sensitive cell is shown. Insulin binds to its transmembrane receptor. Binding induces a conformational change in the β subunit, which in turn activates tyrosine kinase. Once activated, tyrosine kinase phosphorylates intracellular proteins such as insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2). It is through these second messengers that insulin has its effects, activating glucose uptake and stimulating protein synthesis.

insulin concentration are known (Matthews et al., 1985). Estimates of insulin resistance correlate well with estimates from the euglycaemic clamp (R_s = 0.88, $p<0.0001$ (Matthews et al., 1985)) and are reproducible with roughly an 11% intra-individual coefficient of variation (Emoto et al., 1999). In contrast to other methods, HOMA gives an estimate of basal insulin resistance, whereas all other tests provide estimates of stimulated insulin resistance. Limitations of this test are that; it assumes that insulin and glucose are in a steady state at the time of measurements; and the accuracy of this test is dependant on the fact that individuals being tested have a normal insulin secretion, something not found in all patients with T2DM.

In the IVGTT a fixed glucose load is given and glucose and insulin concentrations measured at 5 minute intervals over a 3 hour period. For each patient, estimates of insulin resistance and β cell function are made using curve fitting techniques (Bergman et al., 1979). There are, however, problems with using this technique as for some profiles there is no unique mathematical solution. Since the model used with this test relies on glucose disappearance rate in response to insulin, it is essential to have adequate endogenous insulin secretion. Thus in subjects with diabetes the test needs to be modified by giving a bolus of intravenous tolbutamide (Yang et al., 1987) to augment 2nd phase insulin secretion or by giving a bolus of insulin, 20 minutes into the test (Finegood et al., 1990). Estimates of insulin resistance using this technique correlate well with those from the euglycaemic hyperinsulinemic clamp ($r=0.89$, $p<0.001$ (Bergman et al., 1987). There is a within subject coefficient of variation of roughly 20% (Steil et al., 1986). Limitation of this test are that; it is time consuming and expensive, as 25 blood specimens are taken over a 3 hour period; analysis of the results requires a computer programme; and the lack of standardisation in the methodology used for the IVGTT makes comparisons across studies difficult (Bingley et al., 1992).

The short insulin tolerance test is a simple test in which an estimate of insulin resistance is obtained from the rate of decline in glucose following an intravenous bolus of insulin. Soluble insulin (0.1-0.5 U/kg) is administered intravenously and blood samples (for insulin and glucose) are collected at 2-min intervals for 15

minutes. The short duration of the test avoids the problem of interference from the release of counter-regulatory hormones. The test reflects the combination of suppression of hepatic glucose output and stimulation of peripheral glucose uptake by insulin. Insulin sensitivity is determined by calculating the rate of decline of the log transformed glucose concentrations estimated by linear regression. The test has acceptable reproducibility with a mean within subject coefficient of variation 13%, and between subject coefficient of variation of 26% (Hirst et al., 1993). Insulin sensitivity with this test correlates well with that measured by HOMA in normoglycaemic subjects ($r= -0.61$, $p<0.0001$), but less well in subjects with impaired glucose tolerance ($r=-0.42$, $p=0.05$) (Phillips et al., 1994).

The euglycaemic hyperinsulinemic clamp is now regarded as the gold standard for measurement of insulin sensitivity, (Ferrannini & Mari, 1998). In this insulin is infused at a constant speed to maintain a given insulin concentration such as 80mu/l, whilst glucose is infused at a varied rate to maintain arterial glucose at 5.0mmol/L. The amount of glucose required to maintain this concentration is a measure of insulin sensitivity. The use of radio labelled glucose with this technique allows measurement of the sensitivity of the liver to the inhibitory effect of insulin (switching off of glucose output) and the sensitivity of muscles and adipose tissues to the stimulatory effect of insulin (uptake of glucose into these cells).

Merits of the insulin clamp are that its estimates are free of assumptions, are derived under conditions of steady state, and are reproducible with roughly a 10% intra-individual coefficient of variation (Ferrannini & Mari, 1998). One limitation of this clamp is that it only looks at the effect of insulin on glucose at one glucose concentration. Full dose response curves for *in vivo* insulin-stimulated glucose uptake have been constructed by performing multiple euglycaemic or hyperglycaemic clamps on one individual, but this laborious approach is only feasible in small number of subjects (Rizza et al., 1981). An additional difficulty with these clamps is that insulin sensitivity estimated during insulin administration may not bear a close relationship to that seen in the fasting state where hepatic glucose output and non-insulin-dependent glucose utilization dominate glucose

homeostatis. Despite these limitations the use of the euglycaemic hyperinsulinemic clamp has become prevalent enough to generate databases that can be used to look at the importance of insulin resistance in the pathogenesis of diabetes.

Evidence that insulin resistance is important in the development of diabetes.

There is a large body of evidence that implicates insulin resistance in the pathogenesis of T2DM. The majority of individuals with T2DM are resistant to the action of insulin. This was exemplified in a recent study, which measured insulin sensitivity in a large, tri-ethnic (non-Hispanic whites, Hispanics and African-Americans) cohort of subjects (Haffner et al., 1997) with T2DM. The incidence of insulin resistance was high (83%-96%) in lean as well as obese individuals, regardless of ethnicity.

One way to examine the pathogenesis of T2DM is to study those who are at risk of developing the disease. Individuals with impaired glucose tolerance (IGT) have a ten fold increased risk of developing T2DM when compared to the normal population and have conversion rates estimated between 2-12% per year, depending on the population studied (Ferrannini, 1998). Case control studies have shown that individuals with IGT have a similar degree of insulin resistance to that seen in subjects with T2DM (Bogardus et al., 1984). Furthermore this insulin resistance is independent of obesity (Reavan et al., 1989). Prospective studies in these individuals demonstrate that high fasting insulin concentrations and high 2-hour insulin concentrations after a fixed oral glucose load predict the development of T2DM (Saad et al., 1988).

Having a first degree relative who suffers with T2DM carries a 2-3 fold increased risk of developing T2DM compared to the normal population (Nolan, 2002). These individuals prior to the development of IGT or T2DM demonstrate higher fasting insulin concentration and are more insulin resistant than individuals without a family history. Interestingly the insulin resistance seen in this population is independent of the degree of obesity (Ishikawa et al., 1998). Prospective studies in these individuals also show that high fasting insulin concentrations and high 2 hour insulin

concentration after a fixed oral glucose load predict the development of IGT and T2DM (Lillioja et al., 1993) (Haffner et al., 1996).

Another way to examine the pathogenesis of T2DM is to look at identical twins. Normally the concordance of T2DM in identical twins is between 90-100% (Nolan, 2002). Thus examination of twins non-concordant for T2DM can give us some idea as to what abnormalities precede the development of this disease. Studies reveal that insulin resistance is present from an early age and that the primary abnormality is with insulin stimulated glucose uptake into muscles (peripheral insulin resistance) perhaps caused by a decrease in glycogen synthesis (Gulli et al., 1992).

The final evidence that implicates insulin resistance in the pathogenesis of T2DM comes from two large randomised controlled trials that have shown that it is possible to interrupt the progression from IGT to T2DM by lifestyle interventions (Tuomilehto et al., 2001) (Knowler et al., 2002). In the Diabetes Prevention Programme 3,234 individuals with IGT were randomised to diet, exercise or metformin. The risk of developing diabetes was reduced by 58% in the lifestyle intervention group, members of which maintained daily physical activity (walking or moderate exercise) for 30 minutes per day and lost 5-7% of their body weight. In the group treated with metformin, an insulin sensitising agent, the risk of diabetes was reduced by 31% (Tuomilehto et al., 2001).

In the Finnish Diabetes Prevention Study progression from IGT to T2DM was reduced by a similar degree (58%). Here diet was modified by a reduction in saturated fat and moderate exercise was recommended for 30 minutes per day (Knowler et al., 2002). Common to both of these studies, is a definitive lifestyle intervention resulting in a reduction in acquired insulin resistance, which in turn leads to a reduced incidence of T2DM in a population at very high risk of developing this disease.

How do glucocorticoids cause insulin resistance?

Bierry was the first person to recognise that glucocorticoids were important in glucose metabolism when he demonstrated that adrenalectomised animals became hypoglycaemic (Bierry & Malloizel, 1908). Later when insulin therapy became available for the treatment of diabetes mellitus, a Spanish physician reported that diabetic patients who developed adrenal insufficiency (lack of glucocorticoids) had a reduced need for exogenous insulin, indicating a state of insulin sensitivity (Maranon 1925). Recognition that excessive circulating concentrations of glucocorticoids, whether induced pharmacologically or due to endogenous production, results in insulin resistance confirmed that glucocorticoids are important in determining the action of insulin, i.e. determining the degree of insulin resistance.

It is now recognised that even within the physiological range that glucocorticoids can induce a state of insulin resistance which is seen both in hepatic and extrahepatic tissues. In the liver glucocorticoids increase production of glucose by stimulating the formation of glucose (gluconeogenesis) and by encouraging the breakdown of glycogen (glycolysis) (Rizza et al., 1982). In muscles and adipose tissue glucocorticoids impair both insulin-stimulated glucose uptake and non-insulin stimulated glucose uptake, mainly by reducing glycogen synthesis {Holmang & Bjorntorp, 1992). But how do glucocorticoids induce this state of insulin resistance?

In simplistic terms interference with insulin action (causing insulin resistance) by glucocorticoids can either be generalised affecting all cells and tissues in a similar manner or cell specific, affecting tissues in different ways. Broadly, interference in insulin receptor binding, receptor phosphorylation or second messengers would cause generalised insulin resistance, whereas interference with transporter proteins, enzymes and metabolic pathways would lead to insulin resistance that is tissue specific.

Generalised abnormalities in target organ responses to insulin

In patients with severe insulin resistance, more than 50 mutations of the insulin receptor (Baynes et al., 1997) and 3 mutations of the insulin receptor substrate 1

protein (Yoshimura et al., 1997) have been characterised. However, these mutations are rare and do not explain insulin resistance in the vast majority of patients with T2DM.

Numerous studies have looked at the effect of glucocorticoids on insulin receptor binding but no consensus has emerged. So far, human studies have found that glucocorticoids can decrease insulin receptor binding affinity without decreasing insulin receptor numbers (Kahn et al., 1978) (de Pirro et al., 1980) can decrease receptor number and receptor affinity (Beck-Nielsen et al., 1980) have no effect on affinity or number (Pagano et al., 1983) or can increase receptor number without affecting affinity (Rizza et al., 1982). Where *in vivo* and *in vitro* studies have been carried out simultaneously, they have not been in agreement (de Pirro et al., 1980) (Fantus et al., 1981). It seems reasonable to conclude that the small changes in insulin receptor number or binding affinity would not be sufficient to explain the degree of insulin resistance seen with glucocorticoids. The discrepancies between *in vitro* and *in vivo* observations probably reflect difficulties in controlling for indirect, and potentially compensatory, effects of glucocorticoids. For example, very few experiments have controlled for the hyperinsulinaemia induced by glucocorticoids. When compensatory hyperinsulinaemia was prevented by streptozotocin treatment in rats, glucocorticoid induced changes in insulin receptor number, IRS-1 and phosphorylation were abolished (Giorgino et al., 1993).

Tissue-specific determinants of insulin response

The effect of activation of the insulin receptor differs between tissues. In peripheral tissues, such as fat and skeletal muscle, it depends primarily on altered activity of glucose transporters whereas in the liver it depends on increasing the activity of enzymes controlling glycolysis and gluconeogenesis.

Determinants of peripheral glucose uptake

The first determinant of insulin-dependent peripheral glucose uptake is the availability on the cell membrane of the GLUT 4 transporter, which is expressed mainly in skeletal muscle and increased by insulin. To date, no mutations in GLUT

4 transporters have been reported in patients with insulin resistance (O'Rahilly et al., 1992). The expression of GLUT 4 is increased by glucocorticoids in both skeletal and adipose tissue, but translocation of this transporter to the cell surface in response to insulin and other stimuli (e.g. hypoxia) is inhibited in the presence of glucocorticoids (Oda et al., 1995) (Coderre et al., 1996) (Owen & Cahill, 1973) (Weinstein et al., 1995) (Dimitriadis et al., 1997).

The rate of glucose uptake also depends on the gradient of glucose concentration across the cell membrane. This is influenced both by local delivery of glucose, determined in euglycaemic conditions by blood flow, and by the rate of removal of glucose by phosphorylation or oxidation inside the cell (Figure 1.2). Within the target cell, glucose oxidation is influenced by competing substrates including non-esterified free fatty acids. Randle proposed that free fatty acids might induce insulin resistance by reducing glucose oxidation inside the cell (Randle et al., 1965). This phenomenon is seen during acute administration (Boden, 1997). Furthermore free fatty acids are increased in some subjects with T2DM, especially those who are obese. Acipimox and nicotinic acid, which lower free fatty acid concentrations, also increase insulin sensitivity (Petrie & Donnelly, 1994). Chronic administration of free fatty acids however does not induce insulin resistance (Boden et al., 1995), and free fatty acids may simply be elevated in insulin resistant subjects due to impaired insulin-dependent down-regulation of lipolysis. These findings suggest that elevated free fatty acids could both result from, and contribute to, impaired insulin-dependent glucose uptake.

Increased lipolysis may be important in glucocorticoid induced insulin resistance, since this is reversed by inhibition of lipolysis (Ekstrand et al., 1992) or lipid oxidation (Guillaume-Gentil et al., 1993) (figure 1.2). However, cause and effect are difficult to elucidate because free fatty acids have been reported to influence glucocorticoid receptor binding (Haourigui et al., 1994) (Vallette et al., 1991). Increased lipolysis induced by glucocorticoids may be mediated indirectly, by up-regulation of phenylethanolamine N-methyltransferance (Kennedy et al., 1993), an enzyme expressed in skeletal muscle that converts noradrenaline into adrenaline.

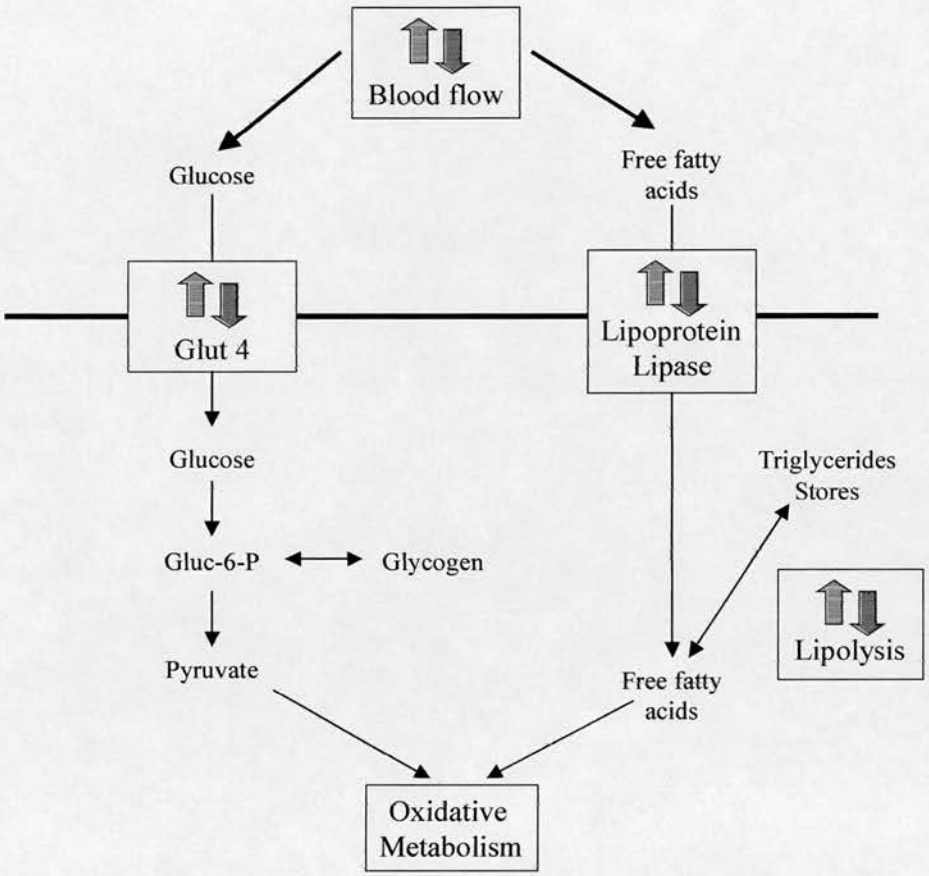


Figure 1.2. Effects of insulin and glucocorticoids on peripheral glucose uptake. A schematic for an archetypal insulin-sensitive cell is shown. In adipocytes, lipogenic pathways predominate whereas in skeletal muscle either oxidative metabolism (of pyruvate or free fatty acids) or glycogen synthesis predominates. GLUT 4 is expressed principally in skeletal muscle and lipoprotein lipase principally in adipose tissue. Actions of glucocorticoids (blue arrows) and insulin (red arrows) are shown either as positive (arrow up) or negative (arrow down) effects. The major effect of glucocorticoids may be to reduce insulin-mediated vasodilatation, reduce translocation of GLUT 4 to the cell surface and enhance local synthesis of adrenaline (see text) thereby increasing free fatty acid competition with pyruvate for mitochondrial oxidative metabolism.

Inhibition of this enzyme ameliorates glucocorticoid induced insulin resistance (Kennedy et al., 1993). Alternatively, effects on lipolysis may be mediated via up-regulation of peroxisome-proliferator-activated γ receptors, for which the insulin-sensitising thiazolidinediones are exogenous ligands. Finally, glucocorticoids may increase circulating free fatty acids by inhibiting lipoprotein lipase (figure 1.2) (Ong et al., 1992).

Baron and others have demonstrated that insulin induces endothelium-dependent vasodilatation, probably mediated by increased nitric oxide synthesis or action (Baron et al., 1995). They have suggested that this action contributes to enhanced glucose uptake in response to insulin and other vasodilator stimuli, particularly in skeletal muscle. Furthermore their model suggests that the impaired endothelium-dependent vasodilatation seen in subjects with the features of the metabolic syndrome (hypercholesterolaemia (Leung et al., 1993), hypertension (Panza et al., 1993) or diabetes mellitus) could both result from, and contribute to, impaired insulin action in skeletal muscle. However, others have found that increased blood flow and glucose uptake during hyperinsulinaemia are dissociated in man (Raitakari et al., 1996).

Glucocorticoids may also influence this determinant of insulin sensitivity. Walker et al have shown that glucocorticoids impair endothelium-dependent vasodilatation in humans *in vivo* (Walker et al., 1995) and therefore, if this is an important mechanism dictating glucose delivery, this may also be a site where insulin action is counterbalanced by glucocorticoids.

Determinates of hepatic glucose release.

The pathways determining the balance between glycogen synthesis and glucose oxidation versus glycogen breakdown (glycogenolysis) and glucose formation (gluconeogenesis) are summarised in Figure 1.3. Abnormalities in hepatic glucose release are most likely to be manifest as increased fasting plasma glucose. Until recently it has been more difficult to measure hepatic than peripheral glucose metabolism in man and the mechanisms of hepatic insulin resistance have thus

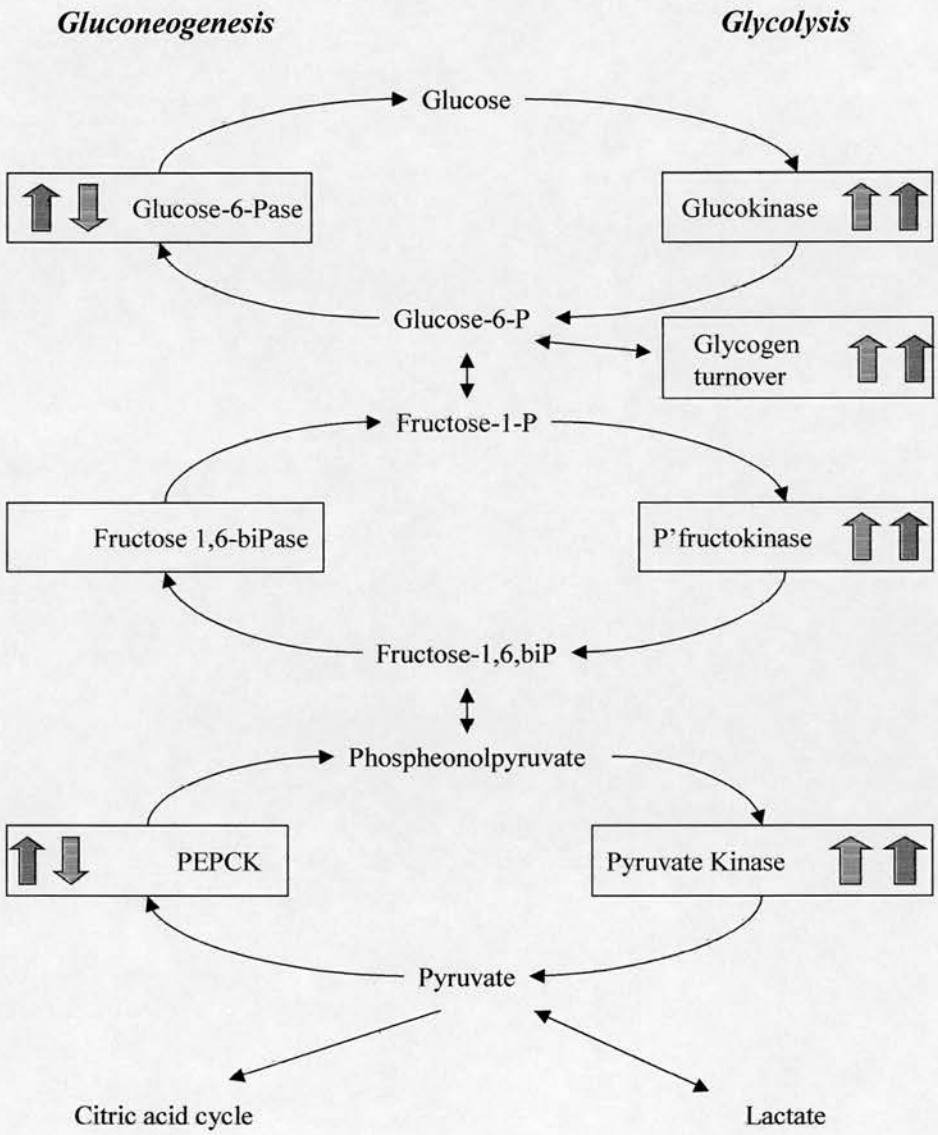


Figure 1.3. Effects of insulin and glucocorticoids on hepatic glucose metabolism The principal metabolic fates of glucose in the liver are shown. Actions of glucocorticoids (blue arrows) and insulin (red arrows) are shown either as positive (arrow up) or negative (arrow down) effects. In some respects, insulin and glucocorticoids oppose each other's actions, particularly on gluconeogenesis (PEPCK) and release of glucose from glucose-6-phophate. In other respects, however, insulin and glucocorticoids do not oppose each other, especially in promoting oxidative glycolysis and increasing turnover between glucose 6-phosphate and glycogen.

remained obscure. One element of insulin signalling which may be specific to the liver, and which has not been accounted for in previous human studies, is the importance of insulin pulsatility (Polonsky et al., 1998). Like other peptide receptors, the insulin receptor responds to specific patterns of change in insulin concentration as well as to the absolute level. Loss of the pulsatile pattern of insulin release may explain the hepatic insulin resistance that occurs prior to the development of T2DM. As has been shown in the earlier section, glucocorticoids interfere with pancreatic insulin secretion, but effects on pulsatility have not been explored.

Contrasting effects of insulin and glucocorticoids on the liver are well characterised in animal models (see figure 1.3) (Dallman et al., 1993). A key effect appears to be the counter-regulation by insulin and glucocorticoids of the rate-limiting enzyme in gluconeogenesis, phosphoenolpyruvate carboxykinase (PEP-CK) (Hanson & Reshef, 1997). However, there is a conflicting literature concerning the effects of glucocorticoids on hepatic glucose metabolism in man. Some groups have found gluconeogenesis to be increased (Rooney et al., 1994) (Pagano et al., 1983) whilst others have not noted any affects following glucocorticoid administration (Malerbi et al., 1988) (Wajngot et al., 1990). These differences may reflect the difficulties of measurement in man, rather than any true discrepancy between species. They could be accounted for by increased glucose/glucose 6-phosphate cycling, which confounds many of the tracer measurements of hepatic glucose output.

Factors which modulate the effect of glucocorticoids on insulin sensitivity

Having described the numerous potential sites of action of glucocorticoids on insulin secretion and sensitivity, I will now address the importance of altered glucocorticoid action in insulin resistance. This requires an understanding of the factors which modulate glucocorticoid action, which are shown in figure 1.4.

Plasma cortisol concentrations

An important determinant of glucocorticoid action is the circulating concentration of cortisol. This is influenced both by the rate of cortisol secretion from the adrenal

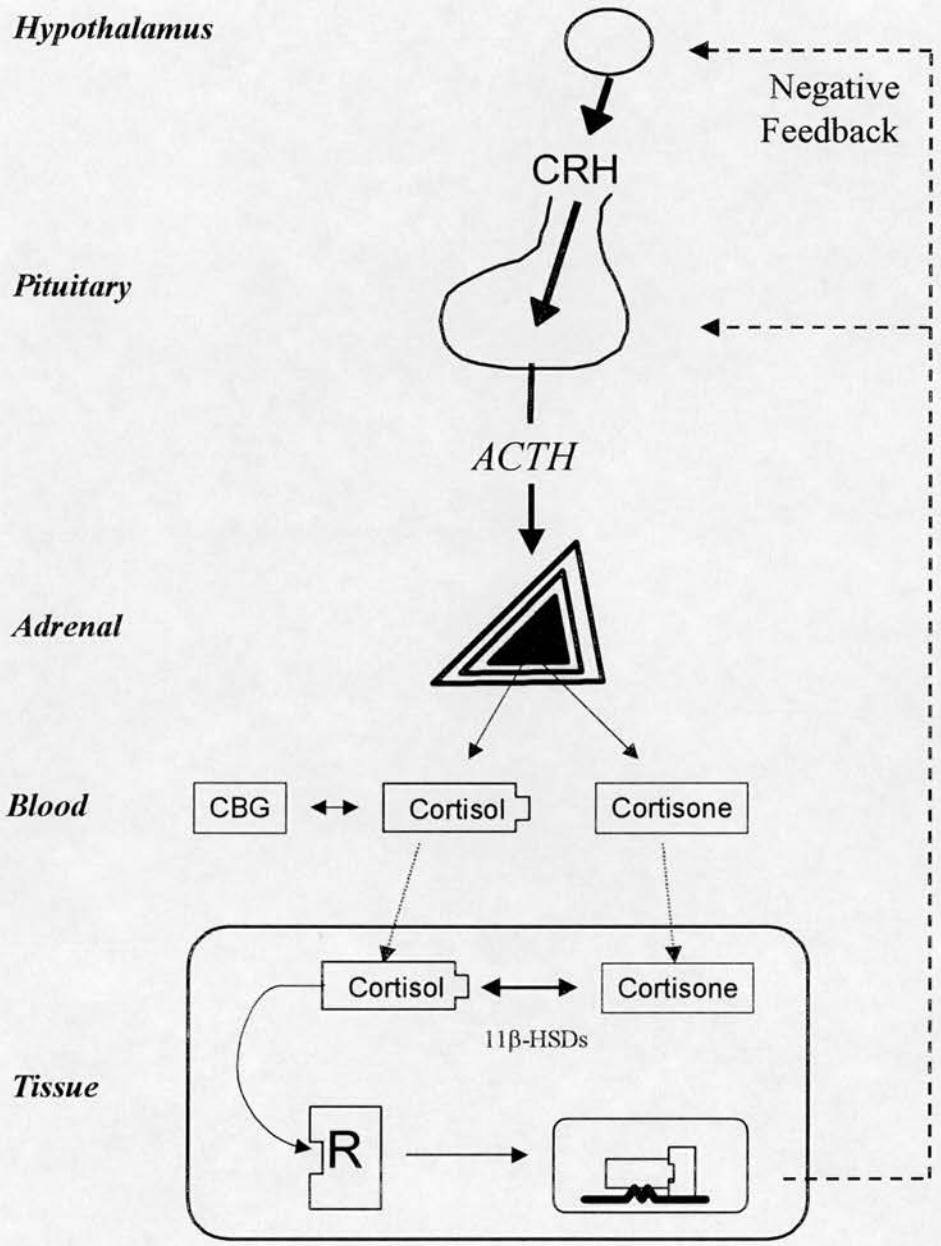


Figure 1.4. Factors determining glucocorticoid action
Schematic indicates the hypothalamic-pituitary-adrenal axis controlling secretion of both glucocorticoids (cortisol) and inactive cortisone. These steroids circulate in similar free concentrations although free cortisol is in equilibrium with a pool of cortisol bound to CBG and albumin. Also shown is a schematic target cell, in which interconversion of cortisol and cortisone by 11β-HSDs dictates access of glucocorticoid to receptors (R) and subsequent regulation of target genes, including those responsible for negative feedback.

cortex, controlled principally by ACTH, and by the metabolic clearance rate of cortisol. Cortisol circulates in plasma in three states; 5-10% circulates unbound and is "free" to cross cell membranes and interact with receptors; 70-75% is bound to cortisol binding globulin (CBG); and 15-20% is bound to albumin. CBG and albumin therefore buffer the free cortisol concentration, but these are saturated within the high physiological range so that there are large excursions in free plasma cortisol concentrations between peaks (in the morning in man and during stress) and troughs (at night in man).

Tissue sensitivity to cortisol

In addition to the influence of changes in circulating cortisol levels, the last decade has seen the recognition of the importance of tissue-specific variations in the mechanisms dictating target organ sensitivity to glucocorticoids. Cortisol can activate either glucocorticoid (type 2 corticosteroid) or mineralocorticoid (type 1 corticosteroid) receptors, and indeed has higher affinity for the latter (Arriza et al., 1987). Glucocorticoid receptors are more widely distributed and act as high capacity, low affinity receptors, which are occupied mostly during the circadian peak of plasma cortisol levels (in the morning in man). By contrast, mineralocorticoid receptors have a more restricted localisation. In some sites, e.g. in hippocampus and hypothalamus, they act as low capacity, high affinity receptors which are occupied during the nocturnal trough of cortisol secretion in man and may be involved in negative feedback control of the HPA axis (Sheppard & Funder, 1987).

In other sites, e.g. the distal nephron, sweat glands and colon, they do not bind cortisol and act as receptors for the much lower plasma concentrations of aldosterone, thereby regulating salt balance (Krozowski & Funder, 1983).

For some time, it was a paradox that mineralocorticoid receptors could bind cortisol in some sites but not in others. This paradox was explained by the activity in aldosterone target sites of an enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which inactivates cortisol by converting it to the metabolite cortisone. When this mechanism is defective, as in a rare congenital syndrome of 11β-HSD2

mutations (Mune et al., 1995), or after administration of the 11β-HSD inhibitor liquorice (Stewart et al., 1987), cortisol gains inappropriate access to mineralocorticoid receptors and induces salt retention, hypokalaemia, and hypertension (Edwards et al., 1988) (Funder et al., 1988).

This model of enzyme-mediated regulation of ligand access to intracellular receptors is not unique to mineralocorticoid receptors. For example, thyroxine is also activated in target tissues to triiodothyronine by 5'-monodeiodinases, testosterone is activated by 5α-reductase to dihydrotestosterone, and similar mechanisms influence activation of vitamin D and retinoid receptors (Stewart & Sheppard, 1992). Recent evidence has suggested that the access of cortisol to glucocorticoid receptors is also regulated by an enzyme, and that this is relevant to the effects of cortisol on insulin sensitivity.

Modulation of insulin sensitivity by 11β-HSD type 1

Before the cloning of 11β-HSD2 (Albiston et al., 1994) (Agarwal et al., 1994), which catalyses the inactivation of cortisol to cortisone, a different isozyme 11β-HSD type 1 (11β-HSD1) had been cloned (Agarwal et al., 1989). This catalyses the same dehydrogenase reaction in solution *in vitro*, but is now recognised to function exclusively as a reductase, reactivating cortisone to cortisol, in whole cells in culture (Low et al., 1994) (Bujalska et al., 1997), in perfused organs (Jamieson et al., 1995), and *in vivo* in man (Walker et al., 1992). 11β-HSD1 is widely distributed throughout the body and is found in the liver, in adipose tissue, and in skeletal muscle. It has been hypothesized that its function in liver is to ensure adequate activation of low affinity glucocorticoid receptors, by reactivating cortisone into cortisol (Figure 1.5). The following evidence supports this hypothesis:

i. Circulating levels of cortisone in man are ~50 nM, and are not protein bound or subject to circadian variation (Walker et al., 1992). This compares with free plasma cortisol concentrations of 50-100 nM in the morning and ~10 nM in the evening. There is therefore an ample supply of substrate for 11β-HSD1 to reactivate to cortisol.

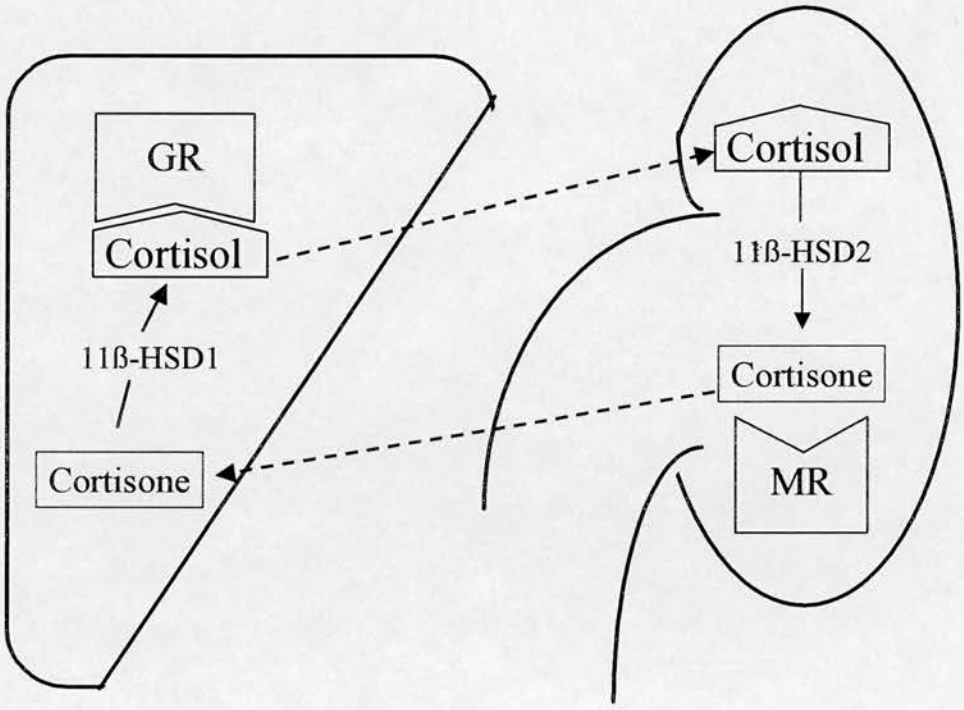


Figure 1.5. Contrasting influence of 11β-HSDs on cortisol sensitivity in the liver and kidney

Predominant conversion of cortisol into cortisone by the dehydrogenase 11β-HSD2 in the kidney results in protection of the local mineralocorticoid receptors (MR). Predominant conversion of cortisone into cortisol by the reductase 11β-HSD1 in the liver results in enhanced activation of glucocorticoid receptors.

ii. The ratio of cortisol/cortisone in the human hepatic vein is 5-fold higher than that in arterial plasma (Walker et al., 1992), confirming that 11β-HSD1 functions as a reductase in the human liver. Furthermore, administration of cortisone by mouth, which is delivered to the liver by the portal circulation, results in high circulating cortisol concentrations but negligible circulating cortisone concentrations (Stewart et al., 1990).

iii. Administration of the liquorice derivative, carbenoxolone, inhibits the conversion of cortisone to cortisol in man (Stewart et al., 1990) and also inhibits hepatic 11β-HSD1 activity in isolated perfused rat liver (Jamieson et al., 1995). Carbenoxolone also results in enhanced whole body insulin sensitivity measured by the euglycaemic hyperinsulinaemic clamp technique but does not alter peripheral insulin sensitivity measured by forearm glucose uptake (Walker, et al., 1995). This suggests that inhibition of hepatic 11β-HSD1 in man results in lower intrahepatic cortisol concentrations which in turn is associated with enhanced insulin-dependent down-regulation of hepatic glucose output.

iv. In rats, oestrogen represses the expression of 11β-HSD1 in the liver (Low et al., 1994). In adrenalectomised rats, oestrogen induces a rise in the gluconeogenic enzyme PEP-CK but in rats with intact glucocorticoid secretion oestrogen suppresses PEP-CK (Jamieson et al., 1998), consistent with enhanced insulin sensitivity due to lower intra-hepatic insulin sensitivity.

v. Transgenic deletion of the 11β-HSD1 gene in mice results in inability to convert 11-dehydrocorticosterone into corticosterone (the equivalent of cortisone and cortisol, respectively, in man) and, despite elevated plasma corticosterone concentrations, is associated with impaired induction of hepatic gluconeogenic enzymes during starvation (Kotelevtsev et al., 1997). Furthermore these animals

show normal hepatic lipid synthesis but an increase in lipid catabolism, resulting in elevated HDL-cholesterol and reduced total cholesterol (Morton et al., 2001).

vi. Selective inhibition of 11β-HSD1 in hyperglycaemic mice, lowers hepatic PEP-CK and glucose-6-phosphatase mRNA, resulting in decreased plasma glucose and insulin concentrations (Alberts et al., 2002).

11β-HSD1 also influences insulin sensitivity in peripheral tissues such as skeletal muscle and fat. Mice with transgenic over-expression of 11β-HSD1 selectively in adipose tissue under the aP2 promoter develop central obesity, insulin resistance, and hyperglycaemia (Masuzaki et al., 2001). Thus specific inhibitors of 11β-HSD1 might provide a useful therapeutic strategy to enhance insulin sensitivity in the liver and adipose tissues in many different syndromes.

Evidence that glucocorticoid activity is increased in subjects with insulin resistance

From the above it is clear that excessive activity of glucocorticoids, whether by increased circulating levels of cortisol or increased tissue sensitivity to cortisol, is a plausible contributor to both insulin resistance and impaired insulin secretion and may thus be important in the pathogenesis of T2DM. This could also explain why hypertension, central obesity, dyslipidaemia, and endothelial dysfunction tend to occur in patients with T2DM.

In addition, glucocorticoid excess *in utero* results in lower birthweight offspring which subsequently exhibit insulin resistance and hypertension (Benediktsson et al., 1993) (Lindsay et al., 1996), so that glucocorticoid excess provides a potential mechanism to explain the association of low birthweight with T2DM and other features of the metabolic syndrome (Barker et al., 1989) (Phillips et al., 1994).

A series of recent studies have examined the relationship between aspects of cortisol secretion and tissue action in these diseases.

Plasma cortisol concentrations: activation of the hypothalamic-pituitary-adrenal axis

Phillips et al have shown that elevated 0900 h plasma cortisol occurs in adult men who were a lower birthweight and is associated with relative hypertension, insulin resistance, glucose intolerance, and hypertriglyceridaemia (Phillips et al., 1998). Similar results have been obtained in other cohorts (Stolk et al., 1996) (Filipovsky et al., 1996). More recent data confirms that these men have evidence of chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis (Reynolds et al., 1998). The rate of cortisol secretion is also increased in young men with a familial predistribution to essential hypertension but not in men with a similar elevation of blood pressure whose parents had low blood pressure (Walker et al., 1998). This suggests that elevated cortisol is an early and perhaps inherited feature of essential hypertension.

Primary activation of the HPA axis may not be responsible for increased cortisol secretion in all circumstances characterised by insulin resistance. The insulin resistance which is associated with obesity is in many ways distinct from insulin resistance in lean subjects, not least because it can usually be reversed by weight loss. Abnormalities of glucocorticoids are also different in lean and obese individuals. The higher plasma cortisol observed in the studies described above appears to co-segregate with insulin resistance but not with obesity. Indeed, plasma cortisol is lower in obese subjects (Ljung et al., 1996) perhaps due to enhanced metabolic clearance of cortisol by the enzyme 5α-reductase, which is expressed in liver and fat (Andrew et al., 1998). The tendency of this enzyme to lower plasma cortisol may result in a compensatory increase in CRH, ACTH and cortisol secretion, which may explain the increased cortisol secretion seen in obesity (Marin et al., 1992) (Pasquali et al., 1993). The same effect may explain the increased drive to adrenal steroidogenesis in the insulin-resistant polycystic ovarian syndrome (Stewart et al., 1990). An alternative mechanism for this increased secretion of cortisol could

be decreased feedback sensitivity to cortisol, which has recently been described by Jessop et al. (Jessop et al., 2001). This again contrasts with changes seen in the HPA of lean insulin resistant men, who show enhanced feedback sensitivity (Reynolds et al., 2001) and inappropriate central drive, as exhibited by impaired habituation of cortisol in response to repeated sampling (Reynolds et al., 2001).

Tissue sensitivity to cortisol

Assessment of glucocorticoid receptor sensitivity in man is difficult. Dexamethasone suppression tests assess central negative feedback suppression of ACTH and cortisol secretion. Although the response to dexamethasone is variably reported as increased or impaired in obesity (Hautanen & Adlercreutz, 1993) (Rosmond et al., 1998), it has not been reported to be abnormal in essential hypertension or lean insulin-resistant subjects.

An alternative test of peripheral glucocorticoid receptor sensitivity *in vivo* involves measuring the intensity of dermal blanching following topical administration of synthetic glucocorticoids. This response is increased in patients with essential hypertension, in young adults with a familial predisposition to hypertension (Walker et al., 1996) and in men with relative glucose intolerance and insulin resistance (Walker et al., 1998). Furthermore, the skin response is increased in healthy subjects who carry a polymorphism of the glucocorticoid receptor gene (Panarelli et al., 1998) which is more common in those with a familial predisposition to hypertension (Watt et al., 1992) and is associated with greater hyperinsulinaemia in obese subjects (Weaver et al., 1992).

Glucocorticoid receptor function can also be measured *ex vivo* in leucocytes. Although these measurements do not relate to the polymorphism associated with increased dermal sensitivity (Panarelli et al., 1998), glucocorticoid receptors have a higher affinity for dexamethasone in leucocytes from subjects predisposed to hypertension (Walker et al., 1998). On the other hand, in established essential hypertension, glucocorticoid receptor binding may be impaired (Mulatero et al., 1997).

These data suggest that glucocorticoid receptor sensitivity may be increased in the metabolic syndrome in peripheral tissues, but not in the central tissues responsible for negative feedback. This inference has remarkable parallels in animal models of the metabolic syndrome. In rats exposed to dexamethasone *in utero* who are born small and develop insulin resistance and hypertension as adults (Benediktsson et al., 1993) (Lindsay et al., 1996), glucocorticoid receptor expression is increased in their liver in association with up-regulation of the gluconeogenic enzyme PEP-CK (Nyirenda et al., 1998). However central glucocorticoid receptor expression is down regulated, explaining why these animals are relatively hypercorticosteronaemic (Levitt et al., 1996). Similarly, men with insulin resistance have now been shown to have normal suppression of plasma cortisol to dexamethasone, but increased expression of the GR in muscle (Reynolds et al., 2002) (Whorwood et al., 2002).

11β-hydroxysteroid dehydrogenases

Cortisol metabolism by 11β-HSDs is also altered in subjects with insulin resistance, although these data are less consistent than the information concerning the glucocorticoid receptor. Insulin is a major inhibitor of 11β-HSD1 expression (Jamieson et al., 1995) (Hammami & Siiteri, 1991), so that it would not be surprising if insulin resistance were associated with differences in the activity of this isozyme. Patients with essential hypertension demonstrate a higher ratio of the metabolites of cortisol to those of cortisone and impaired conversion of cortisol to cortisone (Walker et al, 1998) (Walker et al., 1993) (Soro et al., 1995). However, in patients with polycystic ovarian syndrome, the reverse has been observed in some studies (Rodin et al., 1994).

In the obese Zucker rat, a model for human obesity, abnormalities in cortisol metabolism have been found. These animals have differences in the urinary metabolites of corticosterone (rat equivalent of cortisol) that are consistent with overall balance of whole body 11β-HSDs towards inactive 11-dehydrocorticosterone (rat equivalent of cortisone) (Livingstone et al., 2000). They also show tissue

specific abnormalities in 11β-HSD1, with decreased activity in the liver, normal activity in skeletal muscle and subcutaneous adipose tissue, and increased activity in the omental fat (Livingstone et al., 2000). Similar findings have been found in obese humans with overall 11β-HSDs activity favouring conversion of cortisol to cortisone (Stewart et al., 1999) (Rask et al., 2002), impaired conversion of cortisone to cortisol in the liver and increased re-activation of cortisone to cortisol in omental fat (Rask et al., 2002). Whether similar abnormalities will be found in patients with T2DM remains to be elucidated.

Oral treatment options for patients with type 2 diabetes

The UKPDS study demonstrated that tight glycaemic control can prevent and delay the progression of complications of diabetes (The UKPDS Study Group (33), 1998) (The UKPDS Study Group (34), 1998). In this study the intensive control group aimed to achieve an HbA1c of 7% or less, equivalent to a fasting plasma glucose level of 7.8mmol/l. Regardless of initial response to and mode of treatment, glycaemic control gradually worsened over time. After 3 years only 50% of patients could maintain these targets on monotherapy and this figure fell to 25% at 9 years (Turner et al., 1999). Thus the majority of patients required multiple therapies in an effort to maintain intensive control over time. In many cases this ultimately led to the introduction of insulin. Current options for the tablet of treatment of type 2 diabetes come from three main groups. These work by:

1) Increasing insulin release with sulphonylureas and meglitinides
2) Increasing insulin responsiveness with a biguanide or thiazolidinediones
3) Modification of intestinal absorption of carbohydrate with an alpha – glucosidase inhibitor, or absorption of fat with a lipase inhibitor.

Drugs that increase insulin release.

Sulphonylureas stimulate insulin secretion by increasing response of the pancreatic β-cells to both glucose and non glucose secretagogues (e.g. amino acids). Initially they lower blood glucose concentration by 20%, or decrease HbA1c by up to 2%. The commonest side effect is weight gain which can initiate a vicious circle of

increasing insulin resistance leading to escalation in drug dose and eventually to a relative insulin deficiency. There is some data to suggest that cardiovascular outcome may be worsened in patients taking sulphonylureas (Rytter et al., 1985) (The Digami Study Group, 1997) although further research to confirm or refute this is required.

The meglitinides act in a similar manner to sulphonylureas but have a shorter duration of action. They produce a similar improvement in glycaemic control but are more expensive and have no therapeutic advantages over the older sulphonylureas (Hollander et al., 2001). Long term safety data is not yet available.

Drugs that increase insulin responsiveness

Metformin, a biguanide, improves insulin action by an unknown mechanism. The UKPDS noted that metformin not only improved glycaemic control but also reduced cardiovascular risk in obese patients. This study also found that metformin was associated with the least weight gain when compared to other modes of treatment (The UKPDS Study Group, 1998). Unfortunately many individuals are unable to tolerate metformin or have contraindications to its use.

Thiazolidinediones are the most recent class of drug to be used in type 2 diabetes. They increase insulin sensitivity by acting on muscle and liver to improve glucose uptake and limit glucose production. Although they are known to bind to and activate the transcription factor peroxisome proliferators-activated receptor-gamma, their exact mode of action is still to be elucidated. Unfortunately these drugs have not performed as well as had been anticipated, and although they improve glycaemic control to a small degree (HbA1c improved by <1%), as yet they are not licensed for monotherapy and, unlike other anti-hyperglycaemic agents, they cannot be used with insulin. An additional side effect is that on average individuals gain 4kg in weight with this therapy (Gale, 2001).

Drugs that modify absorption of carbohydrate or fat

Alpha-glucosidase inhibitors prevent the conversion of carbohydrates into monosaccharides in the upper G-I system thus decreasing the absorption of glucose. This increases glucose load in the colon resulting in unwanted gastrointestinal side effects. In those able to tolerate these side effects, only 20%, it can improve HbA1c by up to 0.9% (Catalan et al., 2001).

Orlistat, a lipase inhibitor, reduces the absorption of ingested fat by 30%. In doing so it can lead to an average weight loss of 4kg. This drug has no direct effect on glycaemic control and thus tends to a useful adjunct in the treatment of overweight patients with diabetes. Unfortunately it also has significant gastrointestinal side effects (Miles et al., 2002).

This section demonstrates that present tablet treatment of diabetes is far from ideal. Drug choice is limited, side effects are common, and these drugs only seem to delay an inevitable worsening of diabetes control. Even more disappointing is the fact that no truly effective new class of agent has been found in the last forty years. Research into new therapeutic targets for type 2 diabetes is desperately needed to avoid these individuals ending up on insulin as this in the long term leads to weight gain and further worsening of insulin resistance.

Conclusions

In this introduction I have illustrated the plausibility of a hypothesis that enhanced activity of cortisol contributes to insulin resistance, that it also may impair insulin secretion and thus that may have a significant role to play in the pathogenesis of T2DM. I have also presented evidence to suggest that manipulation of cortisol action may provide a novel therapeutic target to enhance insulin sensitivity and increase insulin secretion. Further work is required to address whether alterations in cortisol secretion and sensitivity are seen in patients with T2DM and to confirm or refute whether manipulation of cortisol action can help in the treatment of this disease.

In this thesis my aims are to determine whether abnormalities in cortisol secretion, metabolism or sensitivity are present in patients with T2DM. I also aim to assess whether manipulation of the activity of 11ß-HSD will improve insulin sensitivity in patients with T2DM.

CHAPTER 2

MEASUREMENT OF CORTISOL METABOLISM AND TISSUE SENSITIVITY TO CORTISOL IN PATIENTS WITH GLUCOSE INTOLERANCE

Introduction

In the previous chapter I explained that enhanced activity of cortisol could be important in the insulin resistance and impaired insulin secretion that is seen in Type 2 Diabetes (T2DM). Interestingly there is a long history in this area. In the early 1940s at autopsies adrenocortical adenomas, non-malignant tumours of the adrenal gland, were reported to be found 16 times more often in patients with diabetes than in individuals without (Russi et al., 1945). Similarly in the mid 1950s patients with diabetes who developed complications were found to have a higher incidence of adrenocortical adenomas and heavier adrenal glands than those who did not (Becker et al., 1954). Whether these pathological abnormalities translate to increased cortisol secretion, metabolism or sensitivity in patients with T2DM is less clear.

Table 2.1 summaries the recent studies that have looked at cortisol activity in patients with diabetes. The majority of these studies have tended to focus on individuals with type 1 diabetes. These have shown increased plasma and urinary free cortisol levels among patients with poor glycaemic control and/or diabetic complications (Couch, 1992) (Dullaart et al., 1995) (Roy et al., 1993) (Dacou-Voutetakis et al., 1998), but these abnormalities were less marked in well-controlled patients without complications (Couch, 1992) (Asfeldt, 1972) (see table 2.1A). A number of older studies looking at patients with both type 1 and type 2 diabetes found less consistent abnormalities (Mortimore et al., 1956) (Kaye et al., 1992) (Hudson et al., 1984) (Huther & Scholz, 1970) (Cameron et al., 1987), but again showed higher plasma cortisol concentrations in those with complications (Lentle & Thomas, 1964) (Tsigos et al., 1993) (table 2.1B). Few studies have included only patients with type 2 diabetes and these did not show altered secretion or circulating levels of cortisol (Serio et al., 1968) (Kerstens et al., 2000). However, obesity (Andrew et al., 1998), gender (Finken et al., 1999) and blood pressure (Walker et al., 1995) affect cortisol secretion and metabolism; these factors were not controlled for in previous studies of patients with diabetes. Moreover, no previous studies have examined tissue responses to glucocorticoids in patients with diabetes, or attempted to dissect tissue-specific changes in cortisol metabolism.

	Demographics				Tests			
	Number	**Gender**	**Type of diabetes**	**Complications of diabetes**	**Plasma cortisol**	**Daily Cortisol rhythm**	**Urinary cortisol production**	**HPA axis**
(Roy et al, 1998)	35	Mixed	Type 1	Yes			Increased UFC	
(Dacou-Voutetakis et al, 1998)	130	Mixed	Type 1	Yes	Higher peak cortisol	Normal	Increased UFC	
(Coiro et al, 1995)	20	Mixed	Type 1	10 with	Higher cortisol than those without complication or controls			Higher ACTH and cortisol response to CRH than those without complication or controls
				10 without	Higher cortisol than controls			Higher ACTH and cortisol response to CRH than controls
(Dullaart et al,. 1995)	12	Mixed	Type 1	6 with	Normal cortisol		Decreased UFC and total metabolites	
				6 without	Normal cortisol		Decreased UFC and total metabolites	
(Roy et al, 1993)	22	Mixed	Type 1	Yes	Higher 9:00 am cortisol		Increased 24 hour UFC	Increased cortisol response to CRH
(Cameron et al,. 1984)	20	Mixed	Type 1	yes		Normal		Reduced suppression to dexamethasone
(Lebinger et al, 1983)	8	Mixed	Type 1	No	Higher peak cortisol	Normal		
(Stacakova et al, 1978)	17	Mixed	Type 1	No			Increased UFC and total metabolites	
(Asfeldt et al, 1972)	19	Mixed	Type 1	Yes	Lower 7:00am cortisol		Normal	

Table 2.1 A. Studies of cortisol secretion, metabolism and sensitivity in patients with Diabetes

	Demographics				Tests			
	Number	**Gender**	**Type of diabetes**	**Complications**	**Plasma cortisol**	**Daily Cortisol rhythm**	**Urinary cortisol production**	**HPA axis**
(Kerstens et al, 2000)	*8*	*Male*	*Type 2*	*No complications*	*Normal*		*Normal*	
(Serio et al, 1968)	*12*	*Mixed*	*Type 2*	*No complications*	*Normal*	*Normal*		*Deceased cortisol response to hypoglycaemia*
(Tsigos et al, 1993)	45	Mixed	Mixed	19 with	Increased cortisol	Higher cortisol at lunch time		
				26 without	Normal	Normal		
(Kaye et al, 1992)	100	Mixed	Mixed	Not mentioned				Normal suppression dexamathasone
(Cameron et al, 1987)	14	Mixed	Mixed	No complications	Higher cortisol			
(Hudson et al, 1984)	30	Mixed	Mixed	Yes				Normal suppression to dexamathasone
(Huther et al, 1970)	17	Mixed	Mixed	No			Increased secretion	
(Lentle et al 1964)	15	Mixed	Mixed	9 with	Higher cortisol	Abnormal rhythm	Increased secretion	Reduced suppression to dexamethasone
				6 without	Higher cortisol	Normal	Normal	Normal suppression to dexamathasone
(Mortimore et al, 1956)	35	Mixed	Mixed	Yes			Normal	

Table 2.1 B. Studies of cortisol secretion, metabolism and sensitivity in patients with Diabetes

For each study information on the demographic variables, gender, diabetes Type, and the presence of complications related to diabetes are shown. All test results are interpreted in comparison with controls. UFC = 24 hour urinary free cortisol, Total metabolites is the sum of all urinary cortisol and cortisone breakdown products and suppression to dexamethasone = cortisol response to a standard 1mg dose of dexamethasone.

Aim

The aim of this study was to examine cortisol secretion, metabolism and sensitivity in nonobese, normotensive, diet-controlled male patients with T2DM or impaired glucose tolerance. The reason for choosing this population was to ensure that measures would not be confounded by those factors known to affect cortisol activity, namely: gender, obesity, blood pressure, poor glycaemic control and diabetic complications (Andrew et al., 1998) (Walker et al,. 1995) (Couch, 1992).

Subjects and Methods

Participants

Twenty five men with T2DM or impaired glucose tolerance (as defined by WHO criteria) were recruited from the diabetes clinics at the Western General Hospital, Edinburgh, UK and 25 normal healthy controls recruited by advertisement. All patients were controlled by diet alone, without oral hypoglycaemic agents or insulin and were free of clinical or biochemical evidence of retinopathy, nephropathy and neuropathy at their last annual review.

Exclusion criteria included:

- Therapy for any other medical conditions – medication may have interfered with measurement being made.
- Major psychiatric disorder – depression is known to affect cortisol activity (Asfeldt, 1969).
- Weight loss >5 kg in the previous 3 months – weight loss of this degree tends to indicate poor glycaemic control.
- Blood pressure >160/90 mmHg – individuals with hypertension have been shown to have abnormalities in cortisol activity (Walker et al., 1996) (Walker et al., 1996) (Walker et al., 1998).
- Body mass index >32 kg/m^2 – individuals with obesity have been shown to have abnormalities in cortisol activity (Andrew et al., 1998).
- Glucocorticoid therapy by any route in the previous 3 months – will affect measures of cortisol activity being made.
- Abnormal renal or thyroid function on biochemical screening – will affect measures of cortisol activity being made (Zumoff et al., 1983).

Control subjects were matched for sex, weight, height, body mass index and blood pressure. Local ethical committee approval and written informed consent were obtained.

Recruitment

Participants were initially contacted by phone and asked it they would like further information about the trial. Those who did were sent written information. After a cooling off period of 2 weeks, they were invited to participate in the trial and a first appointment was arranged for those who were interested.

Protocol (see figure 2.1)

The participants met with the researcher on 5 occasions.

Visit 1 – Baseline measurements – duration 30 minutes

Participants attended on one morning non-fasted. Once formal consent for the trial had been obtained, the researcher performed baseline measurements and blood tests. A brief medical history and examination was made and measurements of sitting blood pressure (using a Takeda UA-751 sphygmomanometer), height and weight were taken. Blood was obtained for full blood count, urea and electrolytes, HbA_1c, liver function tests, and thyroid function tests.

At the end of this visit a timetable for subsequent visits was agreed and recorded on a timetable sheet. A bottle and instructions were provided and the participants asked to collect their urine for a 24 hour period before the next visit. This 24-hour urine specimen sample was used to measure total cortisol metabolites (see below).

Visit 2 – Application of skin test – duration 15 minutes

Participants attended on one afternoon for application of the skin test. Solutions of beclomethasone dipropianate (Sigma Chemical Co.) were prepared in ethanol/water (95:5 vol/vol) at concentrations of 0, 1, 5, 10, 100, or 1000μg/ml (Noon et al., 1996). The six strengths of beclomethasone were labelled A-F by an independent observer

- 1 month	*First contact* Participant contacted by phone, further information sent if interested.
- 2 weeks	*Second contact* Participants contacted by phone, if interested first appointment arranged.
O	*Visit 1 (30 minutes)* Formal consent, history and examination and baseline measurements (blood pressure height and weight and blood specimen) *24 hour urine collection carried out between visit 1 and visit 2*
1 week	*Visit 2 (15 minutes)* Skin test applied and urine collected. *Bandage left on for 17-18 hours, removed an hour before next appointment*
1 week + 1 day	*Visit 3 (40 minutes)* Reading of skin test and skin biopsy if patient agreeable. *Participant asked to remain nil by mouth from 23:00h the night before until 08:30h the next morning*
2weeks	*Visit 4 (15 minutes)* Fasting blood for cortisol, cortisol binding globulin, glucose, and insulin. *Participant asked to take 250μg dexamethasone by mouth at 23:00 h and fasted until attending the following morning at 08:30 h*
3 weeks	*Visit 5 (2 hours and 30 minutes)* Fasting blood for cortisol, then cortisone test.

Figure 2.1. Timetable for study.

with these codes not being revealed until the end of the study. This meant that both the participant and researcher were blinded to the strength of solution being applied.

Between 16:00 and 17:00 h, 50µl of each solution was applied in random order into 6 circles of 20mm diameter on the volar surface of the non-dominant forearm, each site receiving a different solution. After the ethanol had evaporated, all sites were occluded with Saran wrap (Dow), which was removed at 08:00 h the following morning (see figure 2.2).

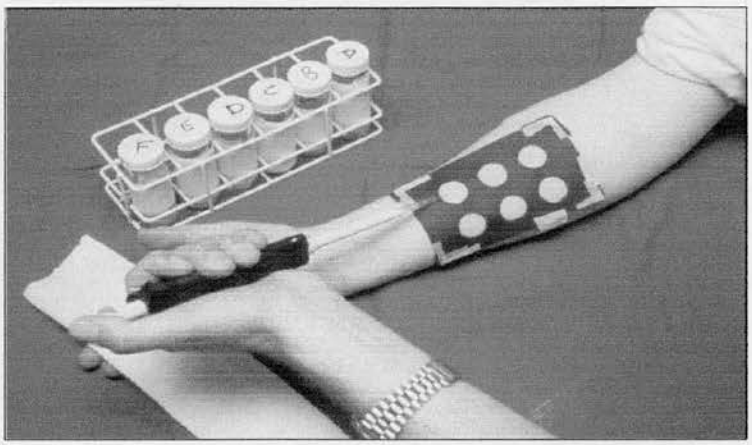
At the end of this visit the 24-hour urine was collected and instructions about what to do with the bandaging were supplied. The total volume of urine was measured and four 50 ml aliquots were stored in a -20°C freezer until analysis (see below).

Visit 3 – reading of skin test and skin biopsy – duration 40 minutes

Having removed the bandaging at 08:00 h each participant attended at 09:00 h for reading of the skin test. The degree of blanching was read by using a reflectance spectrophotometer (Erythemameter, Diastron Ltd). This device measures the ratio of red/green light reflected from the skin surface, called the erythema index (Noon et al., 1996). Because red reflects oxyhaemoglobin concentration and green reflects melanin concentrations, the erythema index corrects for variations in skin colour between individuals. The erythema index for each test site was divided by the erythema index for the site treated with ethanol/water alone to produce a blanching index. The blanching index corrects for the non-specific variations in skin colour that occur in different environments in the same individuals. A lower blanching index indicates more intense blanching and thus a greater sensitivity to glucocorticoids (see figure 2.2).

After reading of the skin tests, participants were asked if they would be willing to undergo a gluteal skin biopsy. Those who consented had a biopsy of skin and subcutaneous fat (2cm x 1cm x 1cm) taken from the gluteal region under local anaesthesia (2% lignocaine hydrochloride; Astra, Herts, U.K.). The biopsy was immediately divided into 3 with 1/3 being frozen immediately at –70 °C for

A)



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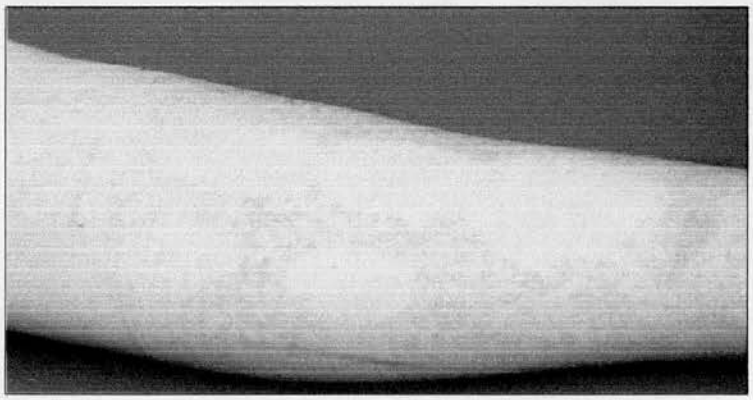

Figure 2.2. Application and reading of skin test.
a) Application:- Solutions of beclomethasone dipropianate were prepared in ethanol/water (95:5 vol/vol) at concentrations of 0, 1, 5, 10, 100, or 1000μg/ml and were labelled A-F by an independent observer. Between 16:00 and 17:00 h 50μl of each solution was applied in random order into 6 circles of 20mm diameter on the volar surface of the non-dominant forearm. After the ethanol had evaporated, all sites were occluded with Saran wrap and left covered until 08:00 h the next day.

b) Reading:- Having removed the bandaging an hour early the degree of blanching was read at 09:00h using a reflectance spectrophotometer. The more intense and widespread the blanching, the greater the sensitivity to glucocorticoids.

measurement of *in vitro* adipose 11β-HSD 1 activity (see below), 1/3 being embedded in paraffin for future measurement of glucocorticoid receptor mRNA by in situ hybridisation (see chapter 3) and 1/3 immersed immediately in cold (4 °C) physiological salt solution (PSS) for later dissection of resistance arteries, which were studied by wire myography (performed by Dr P.W.F. Hadoke see (Mcintyre et al., 2001)).

Visit 4 – Fasting blood test and removal of stitches – duration 15 minutes

Participants attended at 08:30 h having fasted from 23:00 h the previous evening. They lay supine, an intravenous cannula was inserted into the antecubital fossa and blood was taken 30 minutes later for cortisol, cortisol binding globulin (CBG), cholesterol, triglycerides, glucose and insulin. At the end of this visit participants were provided with 0.25mg of dexamethasone and instructions for the next visit. If subjects had had a fat biopsy at the previous visit stitches were removed.

Visit 5 – Dexamethasone suppression test and cortisone test – duration 150 minutes

Participants took 250μg dexamethasone (Decadron; Merck Sharpe & Dohme) by mouth at 23:00 h and fasted until attending the following morning at 08:30 h. An intravenous cannula was sited and 30 minutes later blood was taken for cortisol estimation. Participants then took 25 mg cortisone acetate (Cortisyl; Hoechst Marion Roussel) by mouth and blood was taken for cortisol every 15 minutes for 2 hours.

The difference between the 09:00 h cortisol concentration taken at visit four and that taken at visit five provided a measure of the degree of HPA axis suppression induced by 0.25mg of dexamethasone. The increase in plasma cortisol after the ingestion of cortisone acetate enabled us to look at the hepatic activity of 11β-HSD 1.

In Vitro adipose 11β-HSD 1 activity

Seventeen subjects (5 DM and 12 Controls) consented to return for a 500mg subcutaneous fat biopsy to be taken from the gluteal region under local anaesthesia. Subcutaneous fat was frozen immediately at –70C. After thawing, it was homogenised in Krebs buffer at pH 7.4 and 750μg/ml protein was incubated at 37 °C with NADP (2mM) and 1,2,6,7-$^{3}H_4$-cortisol (100 nM) for 30 hours. Samples were taken at 3, 6, 20 and 30 hours for separation of cortisol and cortisone by HPLC with on-line liquid scintillation detection (Rask et al,. 2001). 11β-HSD1 activity was measured in the dehydrogenase direction (i.e. cortisol to cortisone) rather than the reductase direction (cortisone to cortisol). This was because dehydrogenase activity is more stable than reductase activity in vitro, and because dehydrogenase is the preferred reaction when the enzyme is liberated from its intracellular environment (Seckl & Walker, 2001). Under these conditions, the conversion of cortisol to cortisone is proportionate to the total protein added, and therefore reflects 11β-HSD 1 protein concentrations in the biopsy sample. I performed the biopsies and the experiments for the *in vitro* assessment of 11β-HSD1 activity were kindly completed by Dr Dawn EW Livingstone.

Laboratory analyses

Plasma and urine samples were stored at -80°C and -20°C, respectively.

Radioimmunoassays were used to measure plasma cortisol (McConway & Chapman, 1986), dexamethasone (Cozart Bioscience) and corticosteroid binding globulin (Medgenix diagnostics). Insulin was measured by enzyme immunoassay (Eurogenetics Tasah corporations UK Ltd). Glucose was measured by an enzymatic technique (Cabas Mira Plus, Roche). Ion exchange high performance liquid chromatography was used to measure the HbA_{1c} (Variant 11, Biorad).

Cortisol and its metabolites were measured in urine by electron impact gas chromatography/mass spectrometry following Sep-pak C18 extraction, hydrolysis

with β-glucuronidase, and formation of the methoxime-trimethylsilyl derivatives (Best & Walker, 1997). Epi-cortisol and epi-tetrahydrocortisol were used as internal standards. Intra–assay coefficients of variation were <10% for unconjugated steroid and <20% for total steroids. The limit of detection was 20ηg/sample and peaks were quantified when they exceeded 3 times the background signals. Total cortisol metabolite excretion was calculated as tetrahydrocortisols (THFs) + tetrahydrocortisone (THE) + cortols + cortolones (Zumoff et al., 1974). Relative metabolism by 5α and 5β-reductases were inferred from the 5β-THF/5α-THF ratio. A-ring reduction of cortisol was inferred from the ratios of THFs/cortisol (Ulick et al., 1992) and 5β-reductase activity from the ratio of THE/cortisone. Whole-body equilibrium between cortisol and cortisone, determined by the balance of tissue-specific activities of 11β-reductase and 11β-dehydrogenase activities, was inferred from the ratio of THFs/THE. Renal 11β-dehydrogenase activity was inferred from the urinary free cortisol/cortisone ratio (Best & Walker, 1997) (Faiman & Moorhouse, 1967).

I carried out the measurements for cortisol and CBG. Measurements of insulin, glucose, urea and electrolytes, HbA_1c, liver function tests, thyroid function tests, cholesterol and triglycerides were carried out by the local biochemistry laboratory under the supervision of Mrs Susan Walker. Measurement of dexamethasone and the urinary cortisol and its metabolites were performed by Ms Jill Campbell and Dr Ruth Andrew.

Statistics

Data are expressed as means ± SE. All groups were compared by Student's *t* test apart from the urine data, which were compared by Mann Whitney U test, as these data were not normally distributed. Profiles of cortisol and dermal vasoconstriction were compared by repeated measures ANOVA.

Results

Baseline characteristics

Characteristics of participants are shown in Table 2.2. The groups were well matched for anthropometric, clinical and biochemical variables except that patients with diabetes (DM) had higher fasting plasma glucose, HbA_{1c}, and triglycerides than controls. Cortisol binding globulin and albumin did not differ between the groups so only total plasma cortisol was used in further analysis.

Hypothalamic-pituitary-adrenal axis activity

Fasting morning plasma cortisol (Table 2.2) and total urinary cortisol metabolite excretion rate (Table 2.3) were not different between groups. However, the morning after 250 μg oral dexamethasone, plasma cortisols were lower in patients with diabetes (Table 2.1 and figure 2.3). This could not be attributed to differences in dexamethasone concentrations (Table 2.1).

Cortisol metabolism

Although total cortisol metabolite excretion was not different between groups, there were changes in relative metabolite excretion (Table 2.2). DM patients excreted less unmetabolised cortisol ($p<0.03$) and cortisone ($p=0.07$) and tended to excrete more as 5β-THF ($p=0.07$). As a result, ratios reflecting 5β-reduction of cortisol (5β-THF/cortisol, $p<0.001$) and cortisone (THE/cortisone, $p<0.005$) were increased in DM patients, and there was a trend for increased 5α-reduction of cortisol (5α-THF/cortisol). Absolute excretion of other metabolites, and ratios reflecting 11β-HSD2 (cortisol/cortisone) and overall 11β-HSDs (THFs/THE) were not different.

Hepatic 11β-HSD 1 activity, measured as conversion of orally administered cortisone to cortisol, was impaired in the DM group (Figure 2.4; area under curve 3617 ± 281 nM vs 4475 ± 228 nM; ANOVA $p<0.005$), with an increase in time

	Controls (n=25)	DM patients (n=25)	Student's *t* test P
Age (yr)	59 ± 2	58 ± 2	0.59
Body mass index (kg/m^2)	27.2 ± 0.5	27.6 ± 0.6	0.56
Systolic blood pressure (mmHg)	130 ± 3	131 ± 2	0.66
Diastolic blood pressure (mmHg)	78 ± 2	78 ± 1	0.78
Plasma creatinine (μM)	89.2 ± 2.6	89.8 ± 2.7	0.87
HbA_{1c} (%)	6.0 ± 0.1	6.9 ± 0.2	<0.0001
Fasting plasma glucose (mM)	5.7 ± 0.2	8.2 ± 0.6	<0.0002
Fasting plasma insulin (mU/L)	19.6 ± 4.2	21.4 ± 3.0	0.74
Plasma triglycerides (mM)	2.1 ± 0.2	3.5 ± 0.6	<0.05
Total plasma cholesterol (mM)	5.6 ± 0.2	5.6 ± 0.2	0.92
Plasma Albumin (g/L)	42 ± 0.5	42 ± 0.8	0.50
09:00 h plasma cortisol (nM)	420 ± 30	428 ± 24	0.85
09:00 h plasma cortisol post-dexamethasone 250 μg (nM)	238 ± 20	172 ± 16	<0.01
Plasma cortisol binding globulin (μg/ml)	31.9 ± 1.4	28.8 ± 1.7	0.16
Plasma dexamethasone post-dexamethasone 250 μg (ng/ml)	0.40 ± 0.11	0.41 ± 0.05	0.92

Table 2.2. Clinical Characteristics and biochemistry
Data are mean ± SE

	Controls (n=25)	Hyperglycaemic patients (n=25)	Mann-Whitney U test (P)
Cortisol	100 ± 6	84 ± 9	0.03
Cortisone	112 ± 14	75 ± 6	0.07
5α-tetrahydrocortisol (5α-THF)	1197 ± 153	1196 ± 104	0.66
5β-tetrahydrocortisol (5β-THF)	1036 ± 95	1264 ± 90	0.07
Tetrahydrocortisone(THE)	2218 ± 685	1433 ± 105	0.13
Total cortisol metabolites*	7691 ± 1336	7535 ± 1514	0.80
(5α-THF + 5β-THF)/THE	1.94 ± 0. 19	1.80 ± 0.11	0.47
5β-THF/5α-THF	1.06 ± 0.11	1.19 ± 0.11	0.29
Cortisol/cortisone	1.09 ± 0.08	1.13 ± 0.05	0.97
5β-THF/cortisol	11.0 ± 1.0	16.4 ± 1.0	0.001
5α-THF/cortisol	12.6 ± 1.6	16.5 ± 1.6	0.07
THE/cortisone	15.8 ± 2.2	20.4 ± 1.2	0.005

Table 2.3. Urinary cortisol Metabolites

Results for each steroid are μg/day; other results are ratios; Mean ± SE
*Total cortisol metabolites = 5α-THF + 5β-THF + THE + cortols + cortolones

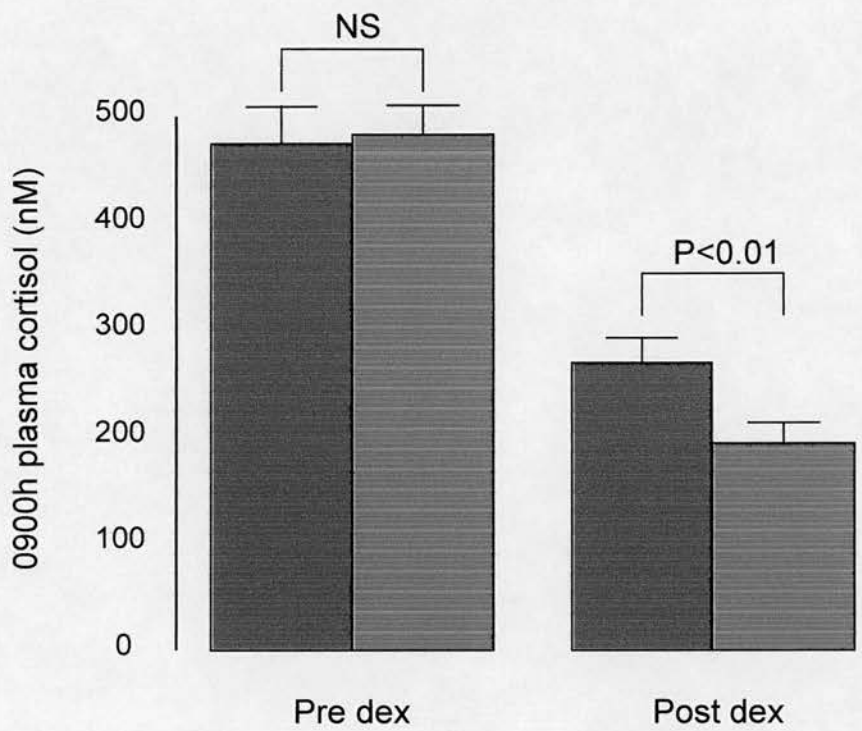

Figure 2.3. HPA axis sensitivity

Plasma cortisol concentrations were measured at 09:00h on two occasions. Once before and once after an oral dose of 250 μg of dexamethasone at 23:00 h. Data are mean ± SE for controls (blue, n=25) and DM patients (Red, n=25). Comparisons by student's *t* tests between the two groups revealed no significant differences at baseline but after dexamethasone the DM group had significantly lower plasma cortisol than the control group.

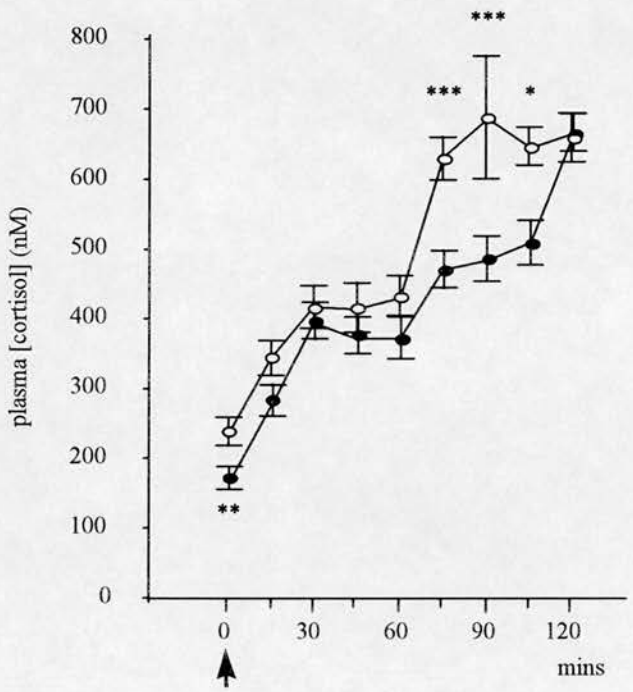

Figure 2.4. ***In vivo*** **hepatic 11β-HSD 1 activity: conversion of oral cortisone to plasma cortisol**

Subjects received oral dexamethasone 250 μg at 23:00 h the previous evening and 25 mg oral cortisone at 09:00 h (time 0; arrowed). Data are mean ± SE for controls (open symbols, n=25) and DM patients (filled symbols, n=25). By repeated measures two-way ANOVA, plasma cortisol was lower in diabetics ($p<0.005$). Asterisks show post-hoc comparisons at each time point by least squares difference tests: * $p<0.02$; ** $p<0.01$; *** $p<0.0001$.

taken to reach maximum plasma cortisol (111 ± 3 min vs 100 ± 4 min, $p<0.05$). By simple regression, there was no relationship between hepatic 11β-HSD1 and any individual urinary cortisol metabolite or ratio. Adipose *in vitro* 11β-HSD 1 activity was no different between the two groups (Figure 2.5 area under the curve 119±21 % controls vs 128±56 % DM, $p=0.8$).

Peripheral tissue sensitivity to glucocorticoids

Dermal vasoconstriction to topical beclomethasone dipropionate was more intense in the DM than the control group (Figure 2.6).

Discussion

This study demonstrates that non-obese normotensive men with hyperglycaemia exhibit abnormalities in cortisol activity. The differences in cortisol metabolism and tissue sensitivity were more striking than any differences in HPA axis function. Specifically, these patients with T2DM or impaired glucose tolerance show:

1) Normal cortisol secretion and circulating levels in the face of enhanced negative feedback sensitivity (as measured with dexamethasone).
2) Enhanced *in vivo* peripheral tissue sensitivity to glucocorticoids (as measured by dermal blanching).
3) Impaired hepatic 11β-HSD 1 activity but normal adipose 11β-HSD 1 activity, suggesting tissue-specific alterations in 11β-HSD 1 activity.
4) Increased relative excretion of A-ring reduced metabolites of cortisol.

These findings suggest that isolated hyperglycaemia is associated with some, but not all, of the changes in cortisol metabolism and action which have been observed in subjects with other features of the Metabolic Syndrome such as hypertension and obesity.

Table 2.1 summaries the recent studies that have looked at cortisol activity in patients with diabetes. The majority of these studies have examined patients with

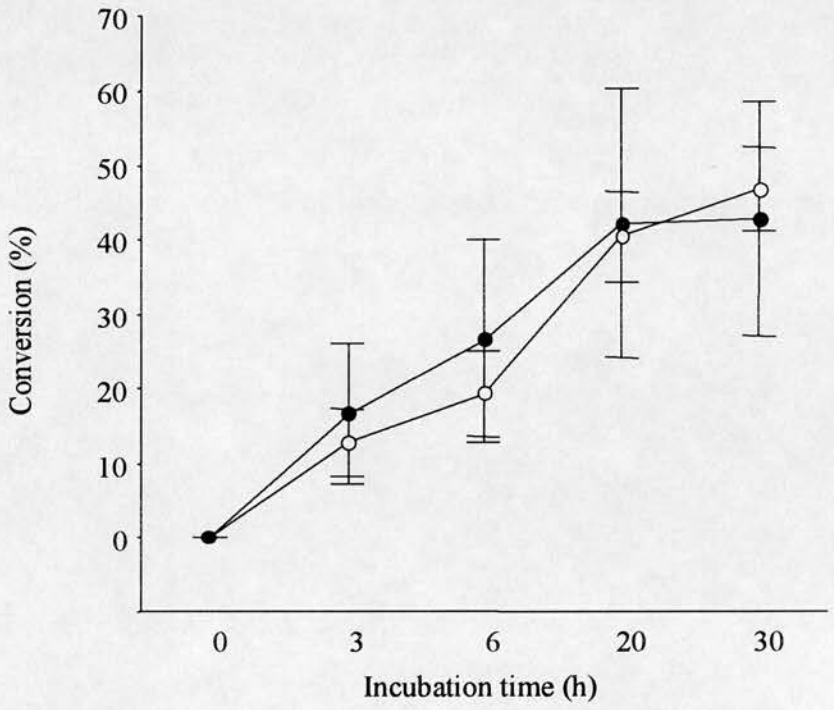

Figure 2.5. ***In vitro*** **11β-HSD 1 activity in subcutaneous fat biopsy.**

Data are mean ± SE for % conversion of cortisol to cortisone at fixed protein concentrations for control subjects (open symbol, n=12) and DM patients (filled symbols, n=5). By repeated measures two-way ANOVA there was no difference between the two groups (p=0.8).

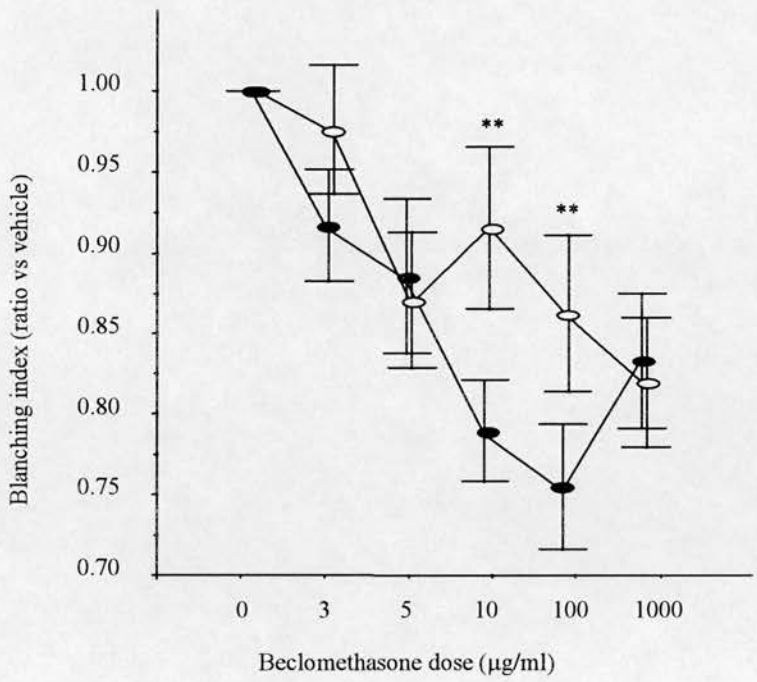

Figure 2.6. Dermal vasoconstriction following topical beclomethasone dipropionate

Blanching index was recorded following overnight topical application of beclomethasone dipropionate. A lower index indicates more intense blanching. Data are mean ± SE for controls (open symbols, n=25) and diabetics (filled symbols, n=25). By repeated measures two-way ANOVA, blanching was greater in diabetics ($p=0.05$). Asterisks show post-hoc comparisons at each dose by least squares difference tests: ** $p<0.01$.

type 1 diabetes or mixed patient populations with few studies including only patients with type 2 diabetes. None of these studies have controlled for factors known to affect cortisol activity, namely: gender, obesity, blood pressure, poor glycaemic control and complications of diabetes (Andrew et al., 1998) (Walker et al., 1995) (Couch, 1992), making interpretation difficult. Studies in patients with type 1 diabetes show increased plasma and urinary free cortisol levels among patients with poor glycaemic control and/or complications of diabetes (Couch, 1992) (Dullaart et al., 1995) (Roy et al., 1993) (Dacou-Voutetakis et al., 1998), but these abnormalities were less marked in well-controlled uncomplicated patients (Couch, 1992) (Asfeldt 1972). Studies looking at patients with both type 1 and type 2 diabetes found less consistent abnormalities (Mortimore et al., 1956) (Kaye et al., 1992) (Hudson et al., 1984) (Huther & Scholz, 1970) (Cameron et al., 1987) but again showed higher plasma cortisol concentrations in those with complications (Lentle & Thomas, 1964) (Tsigos et al., 1993). Studies in patients with type 2 diabetes have not demonstrated any abnormalities in secretion or circulating levels of cortisol (Serio et al., 1968) (Kerstens et al., 2000). No studies to date have examined tissue responses to glucocorticoids in patients with diabetes, or attempted to dissect tissue-specific changes in cortisol metabolism.

The strength of the current study is the careful matching of controls and patients with type 2 diabetes or impaired glucose tolerance, the focus on men only, and the exclusion of patients with obesity, hypertension, and diabetes complications. The aim was to isolate the influence of abnormal insulin action and hyperglycaemia from these confounding effects. This was achieved in so far as the only detected differences in baseline characteristics between patients and controls were in fasting plasma glucose, HbA_{1C}, and triglyceride levels. Fasting insulin levels were not different between groups, consistent with a relative insulin deficiency in the hyperglycaemic patients. In order to achieve this close matching of affected and unaffected groups, it was necessary to select patients with extremely good glycaemic control. As a result, the current study may underestimate effects of hyperglycaemia per se, but nonetheless will detect differences intrinsic to patients with pancreatic β-cell dysfunction.

Other studies have used conventional techniques to assess cortisol secretion, i.e. plasma cortisol concentrations and urinary free cortisol, which are relatively insensitive. Cortisol is secreted in a pulsatile manner throughout the day with higher amplitude and greater frequency in the morning than the evening so that plasma cortisol provides only a brief snap shot of the diurnal pattern. Urinary free cortisol is a small fraction (<5%) of total cortisol metabolite excretion, determined principally by free plasma cortisol clearance. The sum of the urinary metabolites of cortisol in 24h urine, as used in this study, provides a better assessment of 24h secretion of cortisol (Zumoff et al., 1974) although it is still not able to detect changes in diurnal variation of cortisol secretion. Using this method the current study showed that cortisol secretion over 24h is normal in lean patients with type 2 diabetes or impaired glucose tolerance.

The rate of cortisol secretion is controlled by central 'drive' to the HPA axis and by negative feedback suppression by glucocorticoids (see figure 2.7). Dexamethasone suppression of plasma cortisol is the conventional test to examine negative feedback. Previous studies in patients with diabetes have used 1 mg of dexamethasone (Hudson et al., 1984) (Kaye et al., 1992) (Lentle & Thomas, 1964) (Cameron et al., 1984) (see table 2.1) , as is used in clinical practice to detect Cushing's syndrome, and found that in most cases suppression was normal. Interpretation of this test is qualitative rather than quantitative, since the vast majority of controls and patients suppress to below the detection limit for plasma cortisol. In this study we used 250 μg of dexamethasone as an approximate ED_{50} dose in order to quantify more subtle variations in suppression within the 'non-Cushing's' range (Reynolds et al. 2001) (Walker et al., 1996). Using this very low dose test, we have shown that patients with type 2 diabetes have greater sensitivity of the HPA axis to negative feedback. This could not be accounted for by differences in dexamethasone concentrations. Although recent data suggest differences in the feedback response to synthetic and endogenous glucocorticoids in man (Jessop et al., 2001), the finding of normal 24h secretion in the face of this enhanced feedback sensitivity suggests that another factor is driving cortisol secretion.

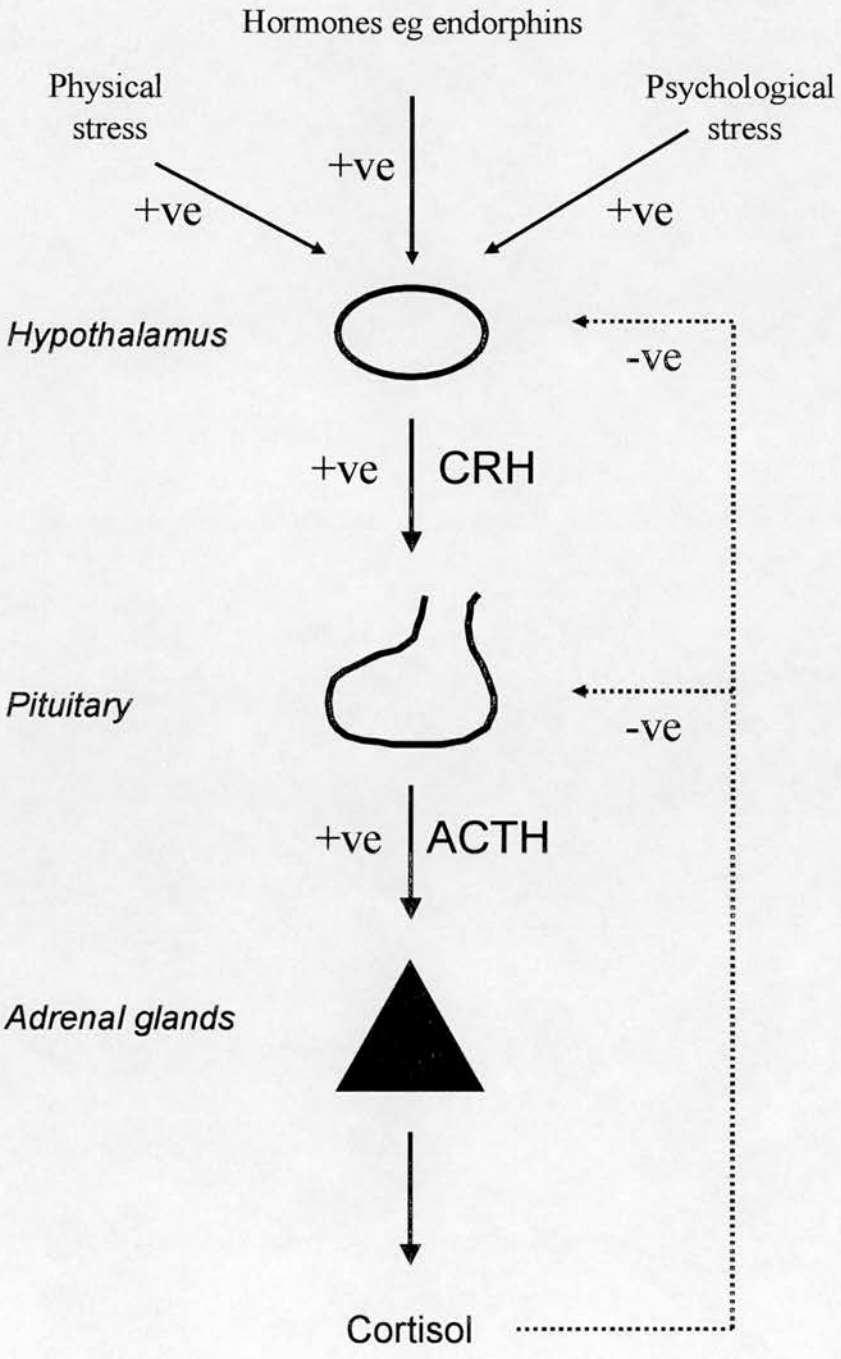

Figure 2.7. Factors influencing cortisol secretion.
Schematic indicating the factors which control cortisol secretion. Cortisol secretion is primarily under the control of the hypothalamic-pituitary-adrenal axis by way of CRH and ACTH. Increasing cortisol concentrations tend to turn off this drive (negative feedback) whereas physical and psychological stress and other hormones can stimulate this drive.

Increased metabolism of cortisol or inappropriate central drive to the HPA axis (figure 2.7) could be the factor driving this cortisol secretion. Studies in obese individuals also show increased cortisol secretion in spite of normal or increased feedback sensitivity (Ljung et al., 1996). Here, increased metabolic clearance of cortisol (Strain et al., 1982), principally by 5α-reductase (Fraser et al., 1999) (Andrew et al.,. 1998) but with increased 5β-reduced metabolites also (Rask et al., 2001), may be a driving force for the increase in cortisol secretion. In this study, we found an increase in the relative excretion of 5α- and, most strikingly, 5β-reduced cortisol metabolites in the absence of obesity in the hyperglycaemic group.

Notably, it has been shown that insulin therapy reduces excretion of 5α-reduced cortisol metabolites (Kerstens et al., 2000). This suggests that peripheral clearance of cortisol is enhanced by mechanisms directly associated with relative insulin deficiency and hyperglycaemia. An alternative explanation is that inappropriate central drive to the HPA, rather than enhanced cortisol clearance, is maintaining cortisol secretion in the face of enhanced feedback in these individuals. This is consistent with the observation that habituation of cortisol in response to repeated sampling is impaired in hyperglycaemic men (Reynolds et al., 2001).

The finding of normal cortisol secretion and circulating cortisol levels in hyperglycaemic patients suggests that if cortisol is to play a role in the pathogenesis of type 2 diabetes, it will be determined by variations in the amount of cortisol made available to peripheral tissue One important determinant of tissue response to cortisol is the extent of metabolism of cortisol within the target tissues by 11β-hydroxysteroid dehydrogenases (11β-HSDs) (see introduction). Two enzymes exist: 11β-HSD 1 which reactivates cortisone to cortisol and serves to maintain adequate exposure of glucocorticoid receptors to cortisol (Seckl & Walker, 2001); and 11β-HSD 2 which converts cortisol to cortisone and prevents cortisol from gaining inappropriate access to mineralocorticoid receptors. Overall, activities of these enzymes can be inferred from the balance of cortisol and cortisone metabolites in urine. These have been measured in previous studies in patients with type 1 diabetes (Dullaart et al., 1995), in whom the ratio of cortisol/cortisone metabolites was lower

than in controls, and type 2 diabetes (Kerstens et al,. 2000), in which there was no difference between relatively obese patients and controls. In this study no differences in overall 11β-HSD activity were found between the controls and individuals with impaired glucose tolerance. However, these urinary ratios are insensitive to tissue-specific changes in 11β-HSD 1 activity (Mortimore et al., 1956).

In obese humans 11β-HSD 1 is decreased in liver (Stewart et al., 1999) but increased in adipose tissue (Rask et al., 2003) (Paulmyer-Lacroix et al., 2002) (Rask et al., 2001). Here, in non-obese hyperglycaemic men, hepatic first pass conversion of cortisone to cortisol was impaired, albeit to a lesser extent than in obese subjects (Rask et al., 2001) (Stewart et al., 1999). However, 11β-HSD 1 activity in gluteal adipose tissue was normal. This could reflect true differences between non-obese hyperglycaemic men and obese men or could be explained by the fact that the biopsies were taken in different regions – those in this study were taken from the gluteal region compared to peri-umbilical biopsies in other studies (Rask et al., 2001). A further confounding factor may relate to the fact that relatively few subjects (17 of the original 50) consented for a biopsy. Nonetheless, there is no trend to suggest that anything approaching the 3-fold differences observed in obesity occur in lean hyperglycaemic subjects.

The mechanism for tissue-specific dysregulation of 11β-HSD 1 in obesity is unknown (Livingstone et al., 1999) (Tomlinson et al., 2001), but these data hint that hepatic dysregulation is related to insulin action while adipose dysregulation is determined by some other factor associated with obesity, or indeed may be a primary mechanism in obesity (Bujalska et al., 1997) (Masuzaki et al. 2001). One possible mechanism for this tissue specific change in 11β-HSD 1 activity is differences in local concentrations of Insulin like growth factors (IGFs). Adminsitraion of GH has been shown to be a potent inhibitor of 11β-HSD 1 activity and even when given in small concentrations can inhibit the conversion of cortisone to cortisol (Toogood et al., 2000). Furthermore changes in local IGF-1 concentrations, free IGF-1 and its binding proteins have been demonstrated in patients with T2DM (Krsek et al., 2003). Thus it is is conceivable that tissue specific changes in 11β-HSD 1 activity could be

explained by alteration in local tissue concentration of GH. Further research is needed to confirm or refute this.

We tested whether variations in A-ring reductase activities might explain variation in hepatic 11β-HSD1 but did not find any correlations. It is intriguing to speculate that down-regulation of 11β-HSD 1 is a compensatory mechanism to protect the liver from glucocorticoid excess in obesity and hyperglycaemia; it may be that the lack of simultaneous increase in adipose 11β-HSD 1 explains why the group of patients studied here are members of an unusual cohort with impaired glucose tolerance but without obesity. Importantly, inhibition of 11β-HSD 1 has been proposed as a therapy to improve metabolic control in diabetes and obesity (Walker et al., 1994) (Bujalska et al., 1997); these data suggest that sufficient 11β-HSD 1 activity exists in patients with type 2 diabetes to make this strategy worthwhile, although it remains to be seen whether inhibition in liver and/or adipose tissue will be most influential (see chapter 4).

Another factor that is important in determining the tissue response to cortisol is the expression and activity of glucocorticoid receptors, which is difficult to measure *in vivo* in man (see chapter 3). Studies comparing sensitivity to synthetic glucocorticoid receptor agonists in different sites suggest that there can be tissue-specific differences. For example, although sensitivity in skin correlates with that in lung (Brown et al., 1991) it may not correlate with that in leukocytes (Panarelli et al., 1998) or in the HPA axis (Ebrecht.M et al., 2000). In this study we show that dermal vascular sensitivity to beclomethasone dipropionate is increased in patients with glucose intolerance. Similar findings have been described in hypertensive and insulin resistant men (Walker et al., 1998) (Walker et al., 1996). This provides circumstantial evidence that glucocorticoid receptors are more readily activated in dermal vessels, although there may be confounding factors influencing the dermal blanching response. Up-regulation of glucocorticoid receptor expression has been implicated in the pathophysiology of insulin resistance in animal models (Nyirenda et al., 1998). Moreover, glucocorticoid receptor mRNA levels in skeletal muscle are elevated in men with insulin resistance (Reynolds et al., 2002) (Whorwood et al.,

2002). These observations suggest that therapeutic strategies to alter glucocorticoid action in key insulin-sensitive target tissues are likely to be especially beneficial in hyperglycaemic patients.

Conclusion

In summary, I have demonstrated that patients with type 2 diabetes or glucose intolerance exhibit abnormalities in cortisol action in the absence of hypertension or obesity. These findings add further weight to the hypothesis that abnormalities in cortisol action may be important in the pathogenesis of T2DM and may explain why abnormalities such as hypertension, obesity and coronary heart disease occur together more commonly than you would expect by chance.

CHAPTER 3

EXPLAINING THE BLANCHING RESPONSE: THE ROLE OF THE GLUCOCORTICOID RECEPTORS.

Introduction

In the previous chapter I demonstrated that dermal vascular sensitivity to beclomethasone dipropionate is increased in patients with T2DM or glucose intolerance. This could be explained by increased access of hormones to the intracellular glucocorticoid receptors (GR), increased GR number, affinity or signalling, or altered sensitivity of the dermal blood vessels to the products of the glucocorticoid target genes. Which of these is the most important in this response is not known.

Increased dermal vascular sensitivity to glucocorticoids has also been described in hypertensive and insulin resistant men (Walker et al., 1998) (Walker et al., 1996). This increased blanching response in patients with hypertension could perhaps be explained by an increase in GR affinity, as GR affinity is greatest in individuals with the highest risk of developing hypertension and lowest in those at the least risk (Watt et al., 1992). In insulin resistant men, this response may be explained by an increase in GR numbers, as their muscles show increased expression of GR (Whorwood et al., 2002) (Reynolds et al., 2002).

In patients with T2DM increased access of hormones to the intracellular GR is unlikely to explain the increased dermal response to glucocorticoids, as overall activity of 11β-HSD 1 and 11 β-HSD 2 are normal (Andrews et al., 2002) and beclomethasone dipropionate is thought not to be metabolised by the 11β HSDs (Walker, 1996). Altered sensitivity of the dermal blood vessels to the products of the glucocorticoid target genes also seems unlikely to explain this response, as *in vitro* studies of subcutaneous vessels show few differences in vasodilation or vasoconstrictive responses between healthy control subjects and patients with T2DM (Andrews, 1999). It thus seems likely that this increased dermal response to glucocorticoid which we have seen in patients with T2DM will be explained by either an increased expression or increased affinity of the GR.

Aims

The aims of this study were to investigate the distribution and concentration of GR in human skin in patients with T2DM and healthy controls. I also aimed to correlate this with the blanching response previously seen (chapter 2).

Subjects and Methods

Participants

All participants who took part in the study described in chapter 2, were asked at visit 3 of the study whether they would be willing to undergo a gluteal skin biopsy. These patients were controlled by diet alone, without oral hypoglycaemic agents or insulin and were free of clinical or biochemical evidence of retinopathy, nephropathy and neuropathy at their last annual review. Those who consented had a biopsy of skin and subcutaneous fat (2cm x 1cm x 1cm) taken from the gluteal region under local anaesthesia (2% lignocaine hydrochloride; Astra, Herts, U.K.). Twenty one subjects (10 DM and 11 controls) consented to the skin biopsy. The biopsy was immediately divided into 3 with 1/3 being frozen immediately at –70 °C for measurement of *in vitro* adipose 11β-HSD 1 activity (see chapter 2), 1/3 being embedded in paraffin for measurement of glucocorticoid receptor mRNA by in situ hybridisation (see below) and 1/3 immersed immediately in cold (4 °C) physiological salt solution (PSS) for later dissection of resistance arteries which were studied by wire myography (performed by Dr P.W.F. Hadoke see (Mcintyre et al., 2001)).

Riboprobes

Human cDNA clone for GR (Seckl et al., 1991) was linearised using the appropriate restriction enzyme. Antisense and sense complementary RNA probes were synthesised from the resultant templates using the appropriate RNA polymerases. Probes were purified on NICK columns (Pharmacia Biotech, Uppsala, Sweden) and checked for size and purity on denaturing polyacrylamide gels.

In Situ Hybridization

The biopsy was embedded in paraffin and horizontal sections (5 μm thick) were cut using a microtome (Leitz GmBH Wetzlar, Germany) and sections placed on 3-aminopropyltriethoxysilane (APES 2%; Sigma, St Louis, MO)-coated slides. Sections were deparaffinised by immersion in Histoclear (2 X 10 mis; Fisher Scientific, Loughborough, Leicestershire, UK).

Histoclear was removed by washing in ethanol (100% x 2 minutes; Merck, Poole, UK). Sections were rehydrated by immersion in graded alcohols (100%, 100%, 95%, 85%, 70%, 50%, 30% ethanol). Ethanol was removed by washing in sodium chloride (0.9%). This was followed by immersion in Triton-X (0.3%; Koch Light, Suffolk, UK) in 1x phosphate-buffered saline (1x PBS for 15 minutes) after which sections were washed twice in 1x PBS (5 minutes). Tissue sections were then digested in trizma–HCl (100 mM, pH 8; Sigma), EDTA (50 mM; Sigma) containing proteinase K (30 minutes, 37°C; Sigma), then washed in glycine (0.1%; Merck) in 1x PBS. Sections were then postfixed in paraformaldehyde (4%; Fisher Scientific), washed in 1x PBS (2 x 5 minutes) followed by acetylation in acetic anhydride (0.25%; Sigma) in triethanolamine (0.1 M, pH 8; Sigma), washed in 1x PBS (1 x 3 minutes), dehydrated in graded alcohols, and air-dried. Sections were incubated with prehybridization buffer made up of diethylpyrocarbonate water, sodium chloride (5 M), trizma base (1 M), 50x Denhardt's (Sigma), salmon testes DNA (Sigma), EDTA (250 mM; Sigma), and yeast tRNA (GIBCO–BRL Products, Paisley, UK) in deionized formamide (50°C x 2 hours; Sigma). Hybridization was then carried out by incubation with ^{35}S-labeled riboprobe (1 x 10^6 cpm) in hybridization buffer containing diethylpyrocarbonate water, sodium chloride (5 M), trizma base (1 M), 50x Denhardt's, salmon testes DNA, EDTA (250 mM), and yeast tRNA in deionized formamide (50°C x 16 hours). After hybridization, sections were washed in SSC (15 minutes) and incubated with Rnase A (100 μg/ml, 37°C for 1 hour; Sigma). Sections were then washed to increasing stringencies to a maximum of 0.1x SSC (60°C for 1 hour). After dehydration through graded alcohols, sections were placed against hyperfilm $ß_{max}$ (2 weeks at 4°C; Amersham) and autoradiographs developed. After this, sections were dipped in photographic emulsion (NTB-2; Kodak, Rochester, NY)

and exposed (4°C for 3 weeks) before being developed and counterstained with hematoxylin and eosin (Sigma).

Measurement of GR

Areas of specific mRNA expression of GR on tissue sections were identified by the appearance of silver grains. Grain counting (SEE-Scan Image Analysis Systems UK) was performed on antisense and corresponding sense sections. Background counts (counts from sense sections) were subtracted from antisense counts and results expressed as absolute grain counts above background. Human liver was used as positive control sections.

Statistics

Data are expressed as means $\pm$ SE or multiples of background. Differences between patient groups were tested by unpaired Student's *t* tests.

Results

Baseline characteristics

Characteristics of the participants are shown in Table 3.1. The groups were well matched for anthropometric, clinical and biochemical variables except that patients with diabetes (DM) had significantly higher HbA_{1c}, and a trend towards higher triglycerides than controls. The 10 patients with DM and 11 healthy controls were representative of both groups studied in chapter 2 (see table 3.1).

Distribution of GR

The results of the in situ hybridisation studies are shown in table 3.2 and figures 3.1, 3.2 and 3.3. In human skin GR mRNA was present in the epidermis, sweat glands,

	DM patients		Controls	
	Previous study (n=25)	This study (n=10)	Previous study (n=25)	This study (n=11)
Age (yr)	58 ± 2	58 ± 3	59 ± 2	60 ± 2
Body mass index (kg/m^2)	27.6 ± 0.8	26.9 ± 0.8	27.2 ± 0.6	28.2 ± 0.6
Systolic blood pressure (mmHg)	131 ± 2	132 +4	130 ± 3	133 + 4
Diastolic blood pressure (mmHg)	78 ± 1	77+2	78 ± 2	79 + 2
Plasma creatinine (%)	89.8 +2.7	90 + 3.7	89.2 + 2.6	91.5 + 4.4
HbA1c (%)	6.9 ± 0.2$^{@@}$	6.6 ± 0.2*	6.0 ± 0.1$^{@@}$	6.1 ± 0.1*
Fasting plasma glucose (mM)	8.2 + 0.6$^{@@}$	6.4 + 0.6	5.7 + 0.2$^{@@}$	5.4 +0.2
Insulin	21.4 ± 3.0	20.1 ± 5.2	19.6 ± 7.0	21.8 ± 7.0
Total plasma cholesterol (mM)	5.6 ± 0.2	5.2 ± 0.3	5.6 ± 0.2	5.2 ± 0.1
Plasma triglycerides (mM)	3.5 ± 0.6$^{@}$	4.2 ± 1.42	2.1 ± 0.4$^{@}$	2.2 ± 0.4
09:00 h plasma cortisol (nM)	438 ± 24	402 ± 30	420 ± 30	415 ± 56

Table 3.1. Clinical Characteristics and biochemistry
Data are mean ± SE
*$p<0.05$ in Student's *t* test DM this study vs Controls this study
$^{@}p<0.05$ and $^{@@}p<0.001$ in Student's *t* test DM previous study vs Controls previous study

	DM	Controls	P value
Epidermis	28 ± 2	26 ± 2	0.40
Sweat Gland	29 ± 4	35 ±3	0.55
Sweat duct	30 ± 4	35 ± 3	0.56
Sebaceous gland	21 ± 2	17 ± 2	0.15
Venules	100 ± 20	91 ± 20	0.59
Arterioles	100 ± 18	81 ± 12	0.32

Table 3.2. Comparison of GR mRNA distribution in the skin between DM patients and healthy controls.
Data are mean ± SE for silver grain counts
Counts are per μM^2 after subtraction of background counts with sense probes.

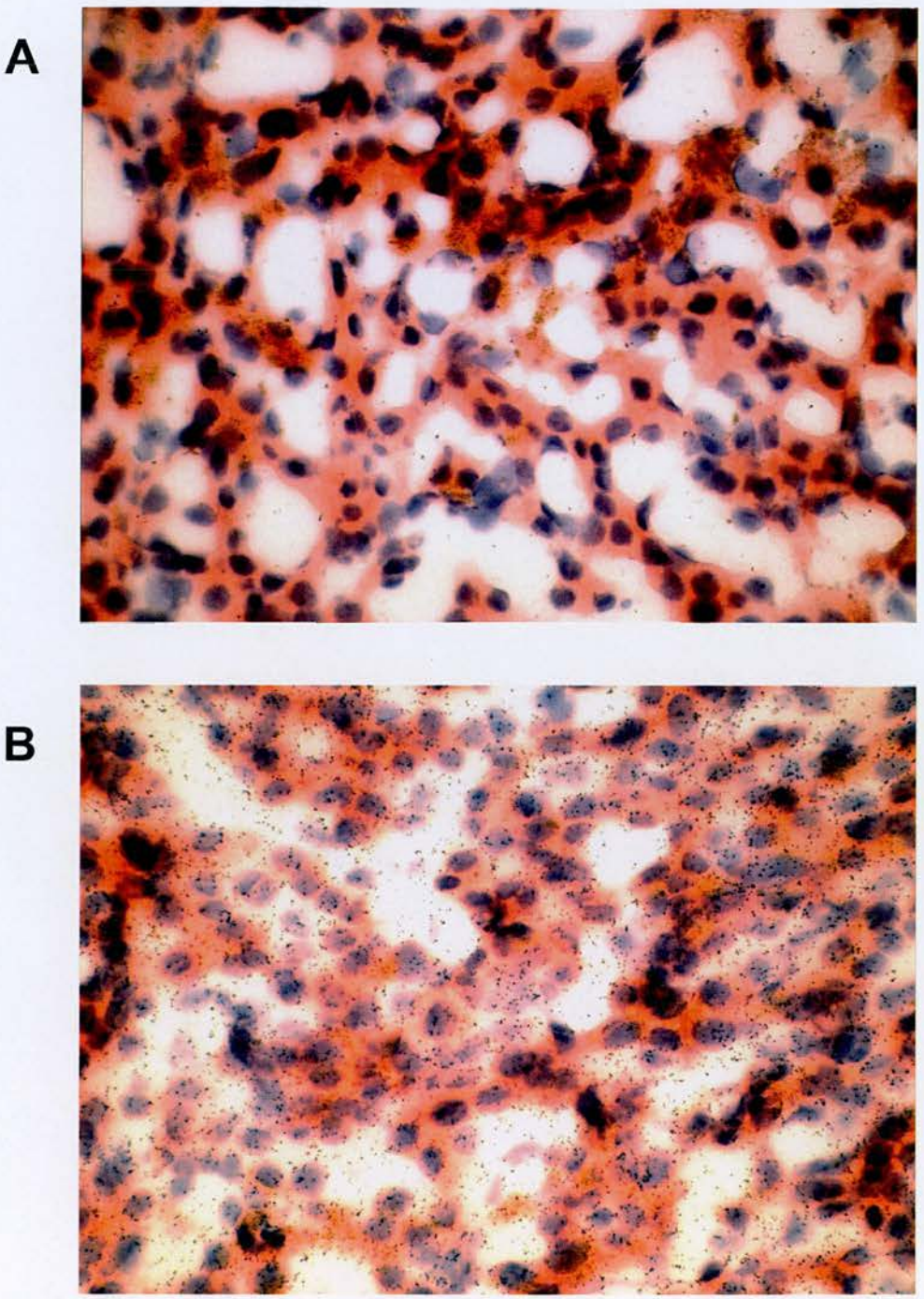

Figure 3.1. Positive controls.
Human in situ hybridization studies for GR in liver. Silver grains denote areas of mRNA expression. (A) sense, (B) antisense. Magnification X40.

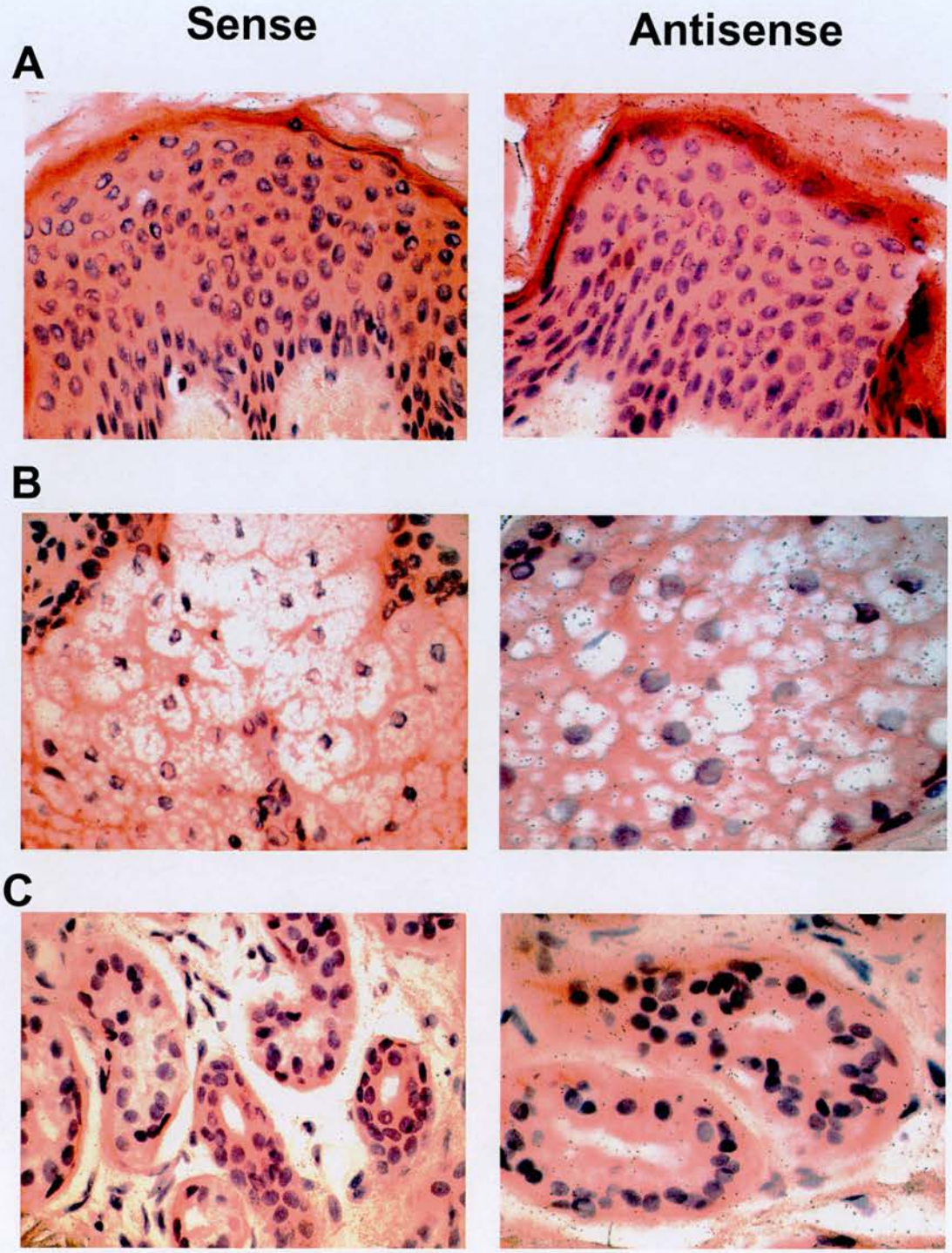

Figure 3.2. Distribution of GR in human skin – 1.
Comparison of Human in situ hybridization studies for GR in skin between sense and antisense slides. Silver grains denote areas of mRNA expression. (A) Dermis, (B) Sebaceous glands, (C) Sweat glands. Magnification X40

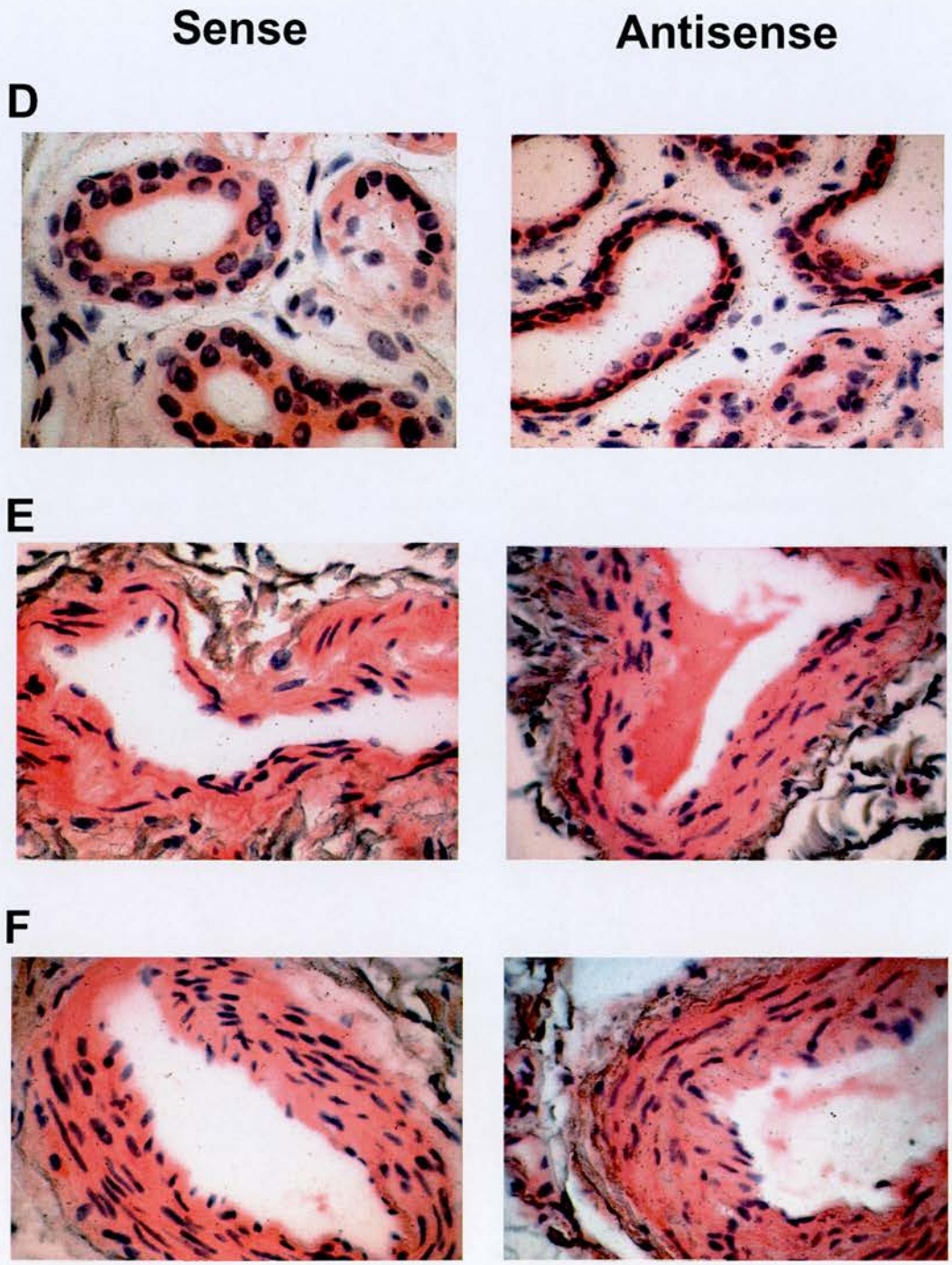

Figure 3.3. Distribution of GR in skin - 2.
Comparison of human in situ hybridization studies for GR in skin between sense and antisense slides. Silver grains denote areas of mRNA expression. (D) Sweat ducts, (E) Venules, (F) Arterioles. Magnification X40

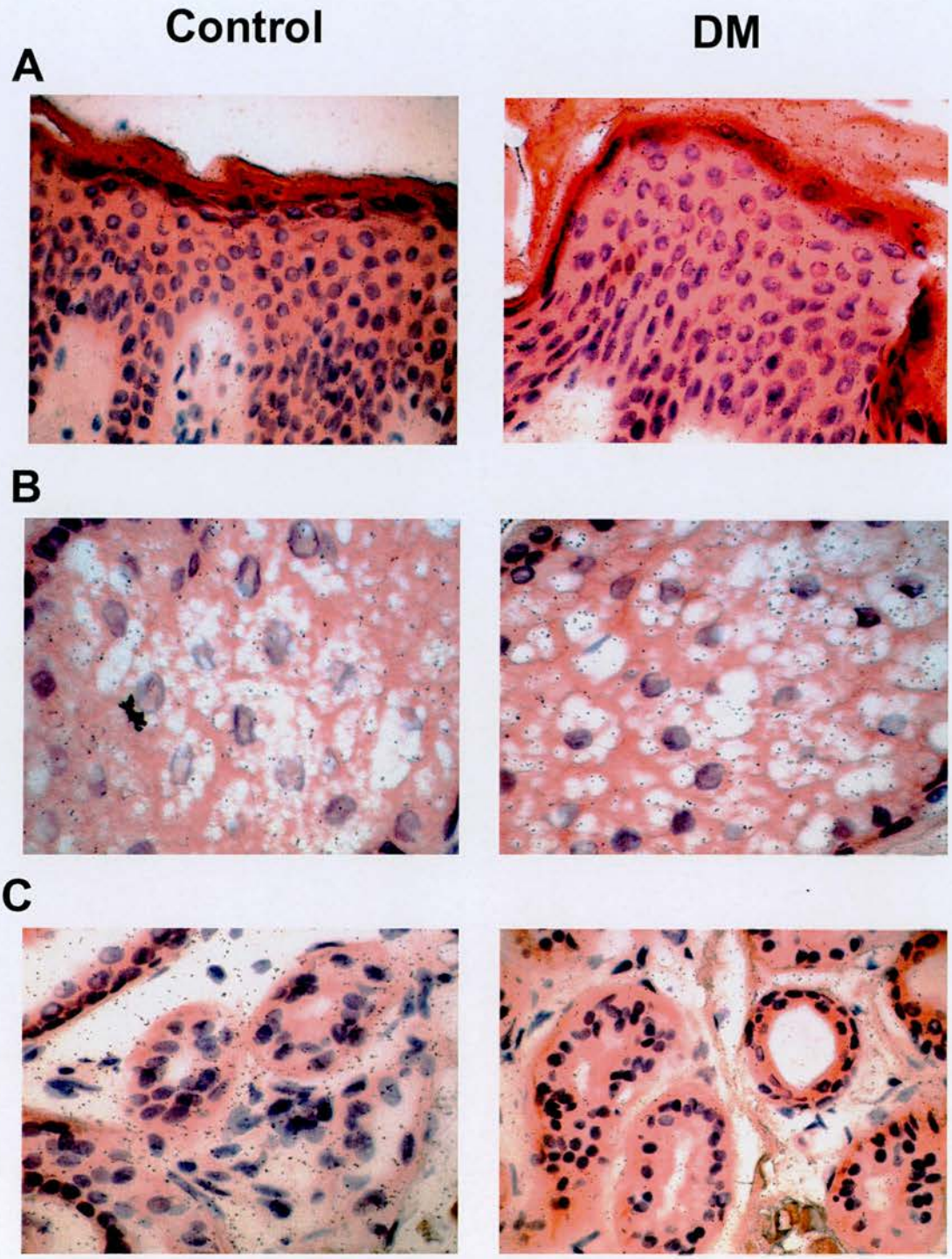

Figure 3.4. Distribution of GR in human skin in DM and healthy controls– 1. Comparison of Human in situ hybridization studies for GR in skin between DM and healthy control slides. Silver grains denote areas of mRNA expression. (A) Dermis, (B) Sebaceous glands, (C) Sweat glands. Magnification X40

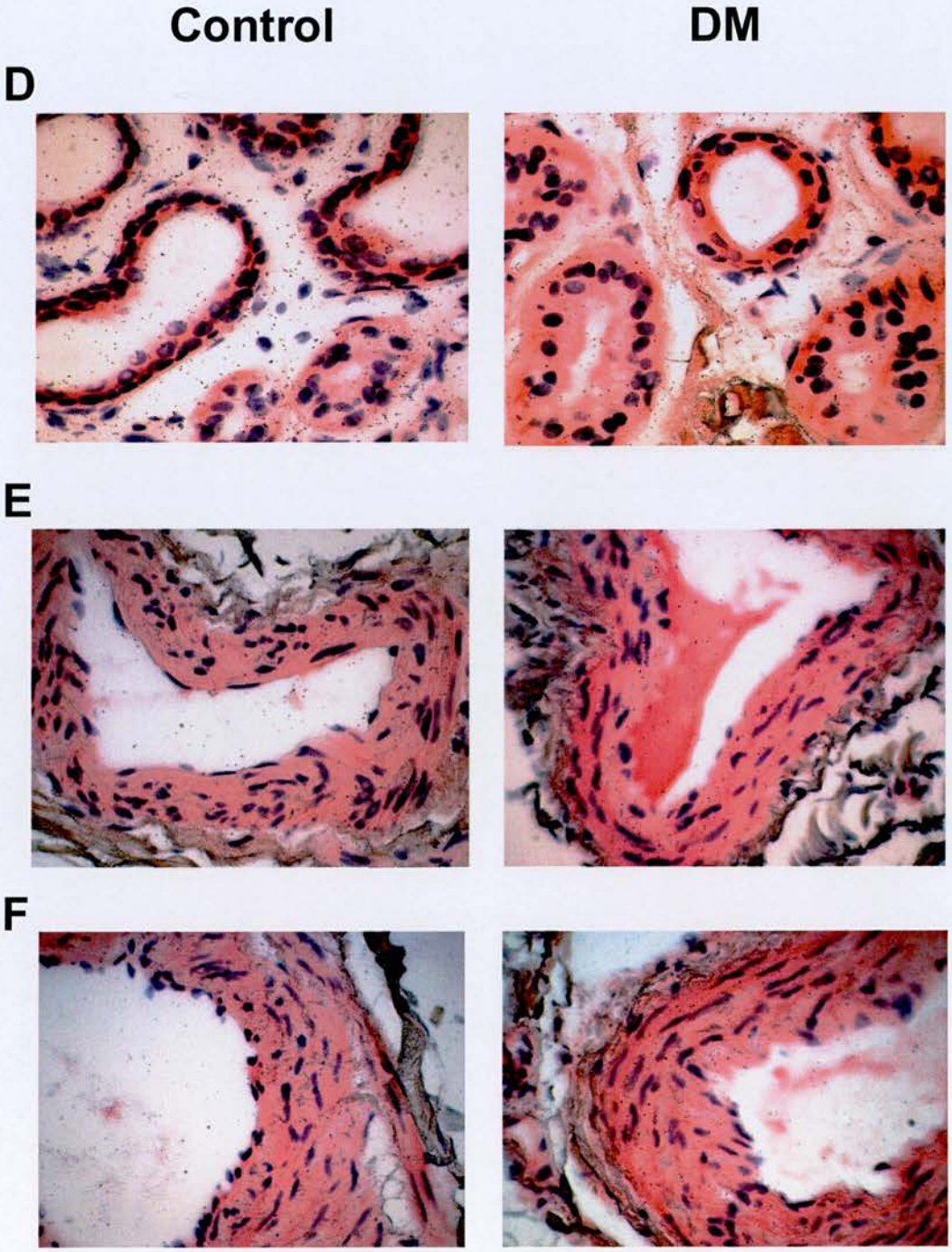

Figure 3.5. Distribution of GR in human skin in DM and healthy controls - 2. Comparison of human in situ hybridization studies for GR in skin between DM and healthy control slides. Silver grains denote areas of mRNA expression. (D) Sweat ducts, (E) Venules, (F) Arterioles. Magnification X40

sweat ducts, sebaceous glands, venules and arterioles. Expression was highest in the arterioles and venules.

Comparison of GR mRNA counts between DM and Controls.

GR mRNA counts for each region in DM and control patients are shown in table 3.2 and figure 3.4 and 3.5. No differences in GR mRNA were seen between DM and controls for any region.

Correlations with in vivo glucocorticoid sensitivity

There was no correlation between GR mRNA concentrations from any region and intensity of skin blanching.

Discussion

This study investigated the distribution of the GR in human skin tissue and attempted to determine whether alterations in expression of this receptor could explain the difference in dermal response to glucocorticoid seen between patients with T2DM or glucose intolerance and healthy controls. In this study we demonstrated expression of GR in the epidermis, sweat glands, sweat ducts, sebaceous glands, venules and arterioles of the skin. However no difference in distribution or mRNA levels of this receptor were found between patients with hyperglycaemia and normal healthy controls. Furthermore there was no correlation between GR concentrations from any region and intensity of skin blanching. These finding suggest that the differences which are seen in the dermal response to glucocorticoids between patients with T2DM or glucose intolerance and healthy controls cannot be explained by alteration in the intracellular concentration of GRs.

Previous studies in rats have demonstrated that GRs are found in both the epidermis and dermis of the skin (Karstilla et al., 1994). In the dermis they are found in hair follicles, sweat glands sebaceous glands and vessels (Karstilla et al., 1994). In humans, studies looking at the localisation of GR have tended to focus on the epidermis, where GRs have been found in the highest concentration in the basal and langerhans cells (Serres et al., 1996). This is the first study which has used in situ

hybridization techniques to look at the expression of the GR in human skin. Previous human studies, using monoclonal antibody techniques, have demonstrated the presence of 11β-HSD1 in human skin in a similar distribution as found in our study (Teelucksingh et al., 1991). This indirectly validates our finding as 11β-HSD1 tends to be found in close proximity to the GR (Monder, 1991).

Although increased dermal sensitivity to glucocorticoids has been shown to predict sensitivity to glucocorticoids in the bronchi, it does not always correlate with that seen in other tissues (Brown et al., 1991) (Walker et al., 1996). In normal healthy male volunteers no correlation has been found between dermal sensitivity to glucocorticoids and sensitivity of blood leukocytes (Panarelli et al., 1998) (Ebrecht et al., 2000). Furthermore individuals with hypertension who show increased dermal sensitivity to glucocorticoids, suppress their plasma concentration of cortisol normally to a 0.25mg dose of dexamethasone, indicating that tissue sensitivity to glucocorticoids must be tissue specific (Walker et al., 1996).

One possible explanation for this variation in tissue responsiveness to glucocorticoids could be tissue specific alterations in GR expression. Rats exposed to dexamethasone *in utero* who are born small and develop insulin resistance and hypertension as adults, show increased glucocorticoid receptor expression in their liver but decreased central glucocorticoid receptor expression (Benediktsson et al., 1993) (Lindsay et al., 1996). Similarly, men with insulin resistance, who show normal suppression of plasma cortisol with dexamethasone, have increased expression of the GR in their muscle (Reynolds et al., 2002).

At the start of this study we felt that the differences seen in the dermal response to glucocorticoids between patients with hyperglycaemia and normal controls would be explained by differences in the expression of the GR. Although we admit that in situ hybridisation is arguably only semi-quantitative, the finding in this study that there is no difference in the distribution or mRNA levels of this receptor between patients with hyperglycaemia and normal healthy controls makes this highly unlikely.

These findings do not exonerate GRs. Alterations in activity or affinity of the GR could still play a role in the differences seen in the dermal response between patients with hyperglycaemia and normal controls. It is now known that there are two isoforms of the GR, GR alpha to which binding of glucocorticoids produces a normal biological response and GR beta to which binding produces no response (Gagliardo et al., 2001) (Hamilos et al., 2001). The in situ hybridisation techniques used in this study are unable to distinguish between these two isoforms, meaning that although there is no difference in total GR expression between the groups there could be differences in the relative ratio of the alpha and beta isoforms. Similarly polymorphisms of the GR gene have been described that can be associated with increased sensitivity to glucocorticoids (Huizenga et al., 1998) (Panarelli et al., 1998) or glucocorticoid resistance in vivo (Weaver et al., 1992) (Brandon et al., 1989). Thus differences in polymorphisms of the GR could explain the differences in the dermal response to glucocorticoid seen between patients with hyperglycaemia and normal controls. Further research is needed to confirm or refute these ideas.

Conclusions

In summary I have demonstrated that in human skin, GRs are expressed in the epidermis, sweat glands, sweat ducts, sebaceous glands, venules and arterioles of the skin. However, differences which are seen in the dermal response to glucocorticoids between patients with T2DM or glucose intolerance and healthy controls cannot be explained by alteration in the intracellular concentration or distribution of these receptors.

CHAPTER 4

THE EFFECT OF THE 11β-HYDROXYSTEROID DEHYDROGENASE INHIBITOR CARBENOXOLONE ON INSULIN SENSITIVITY IN MEN WITH TYPE 2 DIABETES.

Introduction

As mentioned in the introduction, oral treatment for patients with type 2 diabetes (T2DM) is far from ideal. There is little choice of tablets, with only 5 classes of oral hypoglycaemic tablets available for use, and many of these having intolerable side-effects, such as excessive flatulence (α-glucosidase inhibitors), abdominal pain (biguanides) and weight gain (sulpnonlyureas and thiazolidinediones). Furthermore these tablets become less effective over time, due to a gradual increase in insulin resistance combined with a decrease in insulin secretion, resulting in many individuals needing insulin to control their diabetes (UKPDS, 1998). This means that new oral treatments for T2DM are desperately needed.

For many years it has been known that cortisol antagonises the action of insulin, i.e. induces a state of insulin resistance. In the liver it increases glucose production, in the periphery it impairs insulin-dependent glucose uptake and in the brain it stimulates appetite (Rizza et al., 1982) (Holmang & Bjorntorp, 1992) (Stubbs & York, 1991). In addition to these effects on insulin sensitivity, cortisol also inhibits insulin secretion from the pancreatic β-cells (Delaunay et al., 1997) (Ling et al., 1998) (Lambillotte et al., 1997). From this it would seem that drugs designed to lower plasma cortisol concentrations would be ideal for treating patients with T2DM. Unfortunately though, cortisol also plays a key role in regulating growth, salt and water retention, maintaining blood pressure and enabling the body to respond to stressful events meaning that lowering plasma cortisol concentrations could have many serious side-effects. If a way could be found to lower tissue concentrations of cortisol in the liver, fat and muscle without altering plasma cortisol levels this could be a safe and effective drug for treating T2DM.

As has been discussed in previous chapters, the relative activities of 11β-HSD1 and 2 determine the concentration of cortisol seen in tissues. 11β-HSD2 is expressed principally in the distal nephron, where it inactivates cortisol to cortisone, lowering tissue cortisol concentrations and thereby protecting non-specific mineralocorticoid

receptors from cortisol (Edwards et al., 1988) (Funder et al., 1988). 11β-HSD1 is found in the liver, adipose tissue and muscle where it tends to increase tissue cortisol concentrations by reactivating cortisol from cortisone (Seckl & Walker, 2001).

Recent studies in mice have highlighted that the activity of 11β-HSD1 can change drastically without unduly affecting plasma cortisol concentrations. Furthermore, these changes can have profound effects on the metabolic phenotype of the animals. Animals with 11β-HSD1 knockout have normal or marginally increased plasma glucocorticoid levels but cannot regenerate glucocorticoid within cells in liver and adipose tissue. As a result, they are protected from insulin resistance, hyperglycaemia (Kotelevtsev et al., 1997) and weight gain (Morton, N.M., et al., unpublished observations) induced by high fat feeding (Morton et al., 2001). Similarly, down-regulation of 11β-HSD1 expression following administration of oestradiol to male rats is associated with decreased hepatic gluconeogenesis (Jamieson et al., 1998). Conversely, mice with transgenic over-expression of 11β-HSD1 selectively in adipose tissue under the aP2 promoter have increased intra-adipose glucocorticoid concentrations, despite no change in plasma levels (Masuzaki et al., 2001). These animals develop central obesity, insulin resistance, and hyperglycaemia. Mice with transgenic overexpression selectively in the liver under the ApoE promoter also show insulin resistance and hyperlipidaemia (Paterson J, Mullins JJ, Seckl JR et al., personal communication). These finding confirm that pharmacological inhibition of 11β-HSD1 can lower intracellular cortisol concentrations in liver and adipose tissue, without altering circulating cortisol concentrations or responses to stress, and thus is an exciting potential therapy in T2DM.

At present, only relatively non-selective inhibitors of 11β-HSD1 are available for human use. The principal active constituent of confectionary liquorice, glycyrrhetinic acid, and its hemisuccinate derivative carbenoxolone, are potent inhibitors of both 11β-HSD1 and 11β-HSD type 2 (Monder et al., 1989). Inhibition of 11β-HSD2 with liquorice derivatives results in cortisol-dependent mineralocorticoid excess with hypertension and hypokalaemic alkalosis (Stewart et

al, 1987) (Stewart et al., 1990). However, in addition, carbenoxolone inhibits regeneration of cortisol from cortisone by 11β-HSD1 in liver (Stewart et al., 1990) (Andrew et al., 2002) suggesting that it might well improve insulin sensitivity. In healthy men, carbenoxolone was found to improve insulin sensitivity, as measured by an increase in glucose infusion rate during a euglycaemic hyperinsulinaemic clamp (Walker et al., 1994). In this study there was no effect on peripheral glucose uptake, measured by arteriovenous sampling across the forearm, so it was inferred that carbenoxolone was working by lowering intrahepatic cortisol concentrations with a resulting improvement of insulin dependent suppression of hepatic glucose production.

AIMS

The aim of this study was to characterise the mechanism of action of carbenoxolone on insulin sensitivity in healthy men, and quantify its effects for the first time in patients with type 2 diabetes. The selection of patients most likely to respond to 11β-HSD1 inhibition was an important consideration. In obese patients, there is tissue-specific dysregulation of 11β-HSD1 resulting in increased regeneration of cortisol in adipose tissue (Rask et al., 2001) (Rask et al., 2003) (Paulmyer-Lacroix et al,. 2002), but decreased activity in liver (Rask et al., 2001) (Stewart et al., 1999). In contrast, in lean patients with type 2 diabetes we found only a minor decrease in hepatic 11β-HSD1 activity and no change in the enzyme in adipose tissue (see chapter 2) (Andrews et al., 2002). It is now established that carbenoxolone does not inhibits 11β-HSD1 in adipose tissue, but does inhibit in liver (Livingstone & Walker, 2003). For these reasons, we recruited only lean patients with type 2 diabetes and aimed principally to study effects of carbenoxolone in the liver.

Subjects and Methods

Participants

Six men with T2DM (as defined by WHO criteria) were recruited from the diabetes clinics at the Western General Hospital, Edinburgh, UK and 6 normal healthy male controls recruited by advertisement. All patients managed their diabetes by diet alone and were free of clinical or biochemical evidence of retinopathy, nephropathy and neuropathy at their last annual review.

The exclusion criteria included

- Therapy for any other medical conditions – medication may have interfered with measurement being made.
- Major psychiatric disorder – depression is known to affect cortisol activity (Asfeldt, 1969) and thus may have influenced the effect of carbenoxolone.
- Weight loss >5 kg in the previous 3 months – weight loss of this degree tends to indicate poor glycaemic control and possible type 1 diabetes.
- Blood pressure >160/90 mmHg – Carbenoxolone is known to increase blood pressure by effecting 11β-HSD 2 in the kidney (Stewart et al., 1987) (Stewart et al., 1990).
- Body mass index >32 kg/m^2 – individuals with obesity have been shown to have decreased activity of liver 11β-HSD 1 (Rask et al., 2001) (Stewart et al., 1999), the target in this study.
- Glucocorticoid therapy by any route in the previous 3 months – this affects cortisol activity and thus may have influenced the effect of carbenoxolone.
- Abnormal renal or hepatic function on biochemical screening – the major site of action of carbenoxolone is the liver and kidney (Stewart et al., 1990)

Healthy control subjects were matched for, age, weight, height, body mass index and blood pressure. Local ethical committee approval and written informed consent were obtained.

Recruitment

Participants were initially contacted by phone and asked it they would like further information about the trial. Those who did were sent written information. After a cooling off period of 2 weeks, they were invited to participate in the trial and a first appointment was arranged for those who were interested.

Randomisation

Participants took carbenoxolone (100 mg 8 hourly by mouth for 7 days) or placebo in a double-bind randomised crossover trial with phases separated by at least 3 months washout (see figure 4.1). Packets of tablets were made up by the pharmacy and distributed in number order as participants entered the trial. The codes were kept by an independent researcher and not broken until completion of the study.

Protocol (see figure 4.2)

The participants met with the researcher on 7 occasions.

Visit 1 – Baseline measurements – duration 30 minutes

Once participants wishing to enter the trial had been formally consented the researcher performed baseline measurements and blood tests. A brief medical history and examination was made and measurements of sitting blood pressure (using a Takeda UA-751 sphygmomanometer), height and weight were taken. Blood was obtained for full blood count, urea and electrolytes, HbA_1c, liver function tests, thyroid function tests, cholesterol and triglycerides.

At the end of this visit a timetable for subsequent visits was agreed and recorded on a timetable sheet

Visit 2 – provision of tablets – duration 15 minutes

Participants attended at 09:00 h for measurement of weight. They were then issued with their study medication and asked to take the tablets, either placebo or carbenoxolone, three times per day.

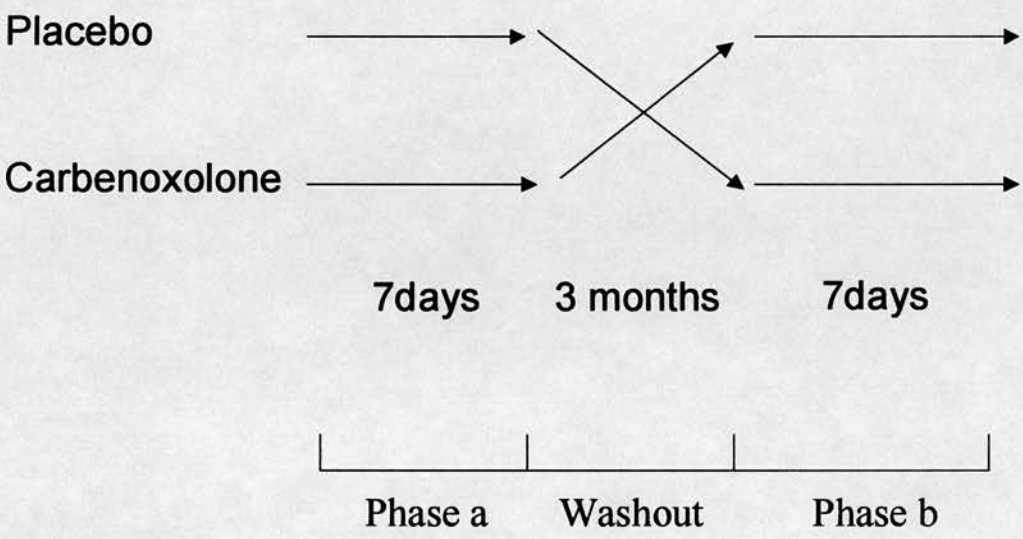

Figure 4.1. Design of trial.
Participants took carbenoxolone (100mg every 8 hours, orally for 7 days) or placebo in a double blind, randomised, cross-over trial with phases separated by a 3 month washout period.

- 1 month	*First contact* Participant contacted by phone, further information sent if interested.
- 2 weeks	*Second contact* Participants contacted by phone, if interested first appointment arranged.
O	*Visit 1 (30 minutes)* Formal consent, history and examination and baseline measurements (blood pressure height and weight and blood specimen)

Randomisation of patients

1 week	*Visit 2 (15 minutes)* Participants weighed and provided with tablets
1 week + 4 day	*Visit 3 (15 minutes)* Participants weighed, blood taken for U+Es and meal choice for visit 4 made.

Participant asked to attend at 17:00 h the night of the 7th day of each phase.

2 weeks	*Visit 4 (20 hours)* Standard meal at 17;30h, thereafter only oral intake was water. 22:00 h commencement of euglycaemic clamp study.

3 month washout period

14 weeks	*Visit 5 (15 minutes)* Participants weighed and provided with tablets
14 week + 4 day	*Visit 6 (15 minutes)* Participants weighed, blood taken for U+Es and meal choice for visit 7 made.

Participant asked to attend at 17:00 h the night of the 7th day of each phase.

16 weeks	*Visit 7 (20 hours)* Standard meal at 17:30h, thereafter only oral intake was water. 22:00 h commencement of euglycaemic clamp study.

Figure 4.2. Timetable for study.

Visit 3 – further measurements – duration 15 minutes

Four days later participants returned for measurement of weight, blood pressure and plasma electrolytes, as carbenoxolone can elevate blood pressure, lead to sodium retention and induce hypokalaemia. If the participant developed hypertension (blood pressure >160/100), signs of heart failure (>5% gain in weight), or hypokalaemia (potassium <3.2 mmol/l) they were withdrawn from the study

Visit 4 – measurement of insulin sensitivity – duration 20 hours

On day seven participants returned to the Clinical Research Facility for an overnight stay with measurement of insulin sensitivity by a hyperinsulinaemic normoglycaemic clamp the next morning (see protocol). At the end of this visit participants underwent a washout period for 3 months and then repeated visits 2- 4 using placebo/carbenoxolone (figure 4.1).

Compliance

Compliance with study medication was monitored by tablet counting and by measuring plasma carbenoxolone levels in samples obtained at 07:00 h on the day of the clamp study. No subjects had to with draw due to adverse effects.

Euglycaemic clamp protocol (figure 4.3)

Participants were asked to attend the Clinical Research Facility at 17:30h for a standardised meal. This is was to ensure that the amount of carbohydrate and fat taken before the clamp was similar in all groups, as variation in levels of carbohydrate and fat taken prior to a hyperglycaemic normoglycaemic clamp have been shown to affect insulin sensitivity (Robertson et al., 2002). After this standard meal their only oral intake was water.

Cannulae were placed in an antecubital vein for infusions and retrogradely in a contralateral dorsal hand vein; the hand was kept in a hot box for arterialised blood sampling. The clamp was divided into three phases;

Phase 1. From 22:00-07:30h, an overnight clamp was employed to ensure that controls and T2DM patients started the hyperinsulinaemic clamps with similar

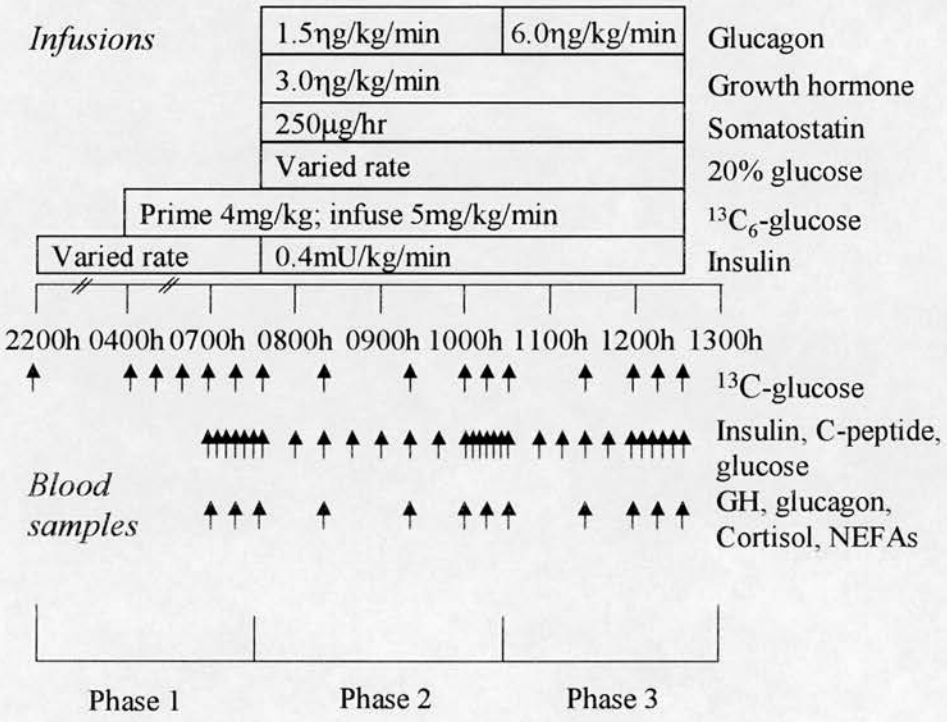

Figure 4.3. Protocol for clamp study.
The clamp is divided into 3 phases: Phase 1 - from 22:00-07:30h, an overnight clamp was employed to ensure that controls and DM patients started the next phase with similar plasma glucose concentrations, Phase 2 - from 07:30-10:30h, a hyperinsulinaemic normoglucagonaemic normoglycaemic clamp was performed and phase 3 – from 10:30-12:30h a hyperinsulinaemic hyperglucagonaemic normoglycaemic clamp was performed. Arrows indicate the timing of the blood sampling.

plasma glucose concentrations. Blood glucose was measured at least every 15 minutes and intravenous insulin was administered at variable rate, if required, to maintain glucose at 5.0mM. From 04:00h, $^{13}C_6$-glucose was infused (at 4 mg /kg/h after priming with 4 mg/kg). In the last half hour of this phase measurements of basal parameters were made.

Phase 2. From 07:30-10:30h, a hyperinsulinaemic normoglucagonaemic normoglycaemic clamp was performed with infusions of insulin (0.4mU/kg/min), somatostatin (0.25mg/h), glucagon (1.5ηg/kg/min), growth hormone (3ηg/kg/min) and 20% glucose. The 20% glucose infusion rate was varied to maintain arterialised blood glucose at 5.0mM.

Phase 3. From 10:30-12:30h, a hyperinsulinaemic hyperglucagonaemic normoglycaemic clamp was performed by increasing the glucagon infusion rate from 1.5ηg/kg/min to 6.0ηg/kg/min while maintaining other infusions.

In addition to frequent samples for bedside blood glucose monitoring, blood samples were obtained as indicated in Figure 4.3, which were immediately centrifuged and the plasma frozen and stored at -80°C until analysis.

Laboratory analyses

Enzyme immunoassays (from Eurogenetics Tasah corporations UK Ltd) were used to measure plasma insulin, growth hormone, and C-peptide. Electrolytes were measured on a Vitras 950 (Ortho clinical diagnostics) and glucose on a Cabas Mira Plus (Roche). Triglycerides, total cholesterol and HDL cholesterol were measured using ELISA kits (TG, CHOL and HDL C-plus, respectively)(Roche Diagnostics, Mannheim, Germany). HbA_{1C} was measured by ion exchange high performance liquid chromatography (Variant 11, Biorad). Radioimmunoassays were used to measure cortisol (McConway & Chapman, 1986) and glucagon (Orskov et al., 1968). Free fatty acids were measured by a colorimetric technique (Wako, Neuss, Germany). Carbenoxolone was measured by high pressure liquid chromatography

with ultraviolet detection (at 254nm) using 18α-glycyrrhetinic acid as internal standard.

Enrichment of glucose isotopomers was analysed as its acetylated di-*0*-isopropylidene derivative (Hachey et al., 2001) using a gas-chromatograph quadrupole mass spectrometer (Voyager, Thermoquest, Manchester, UK). Electron impact ionisation was used with selective monitoring of masses 287-293. Enrichment of lactate isotopomers was analysed as its propyl-amideheptafluorobutyric acid using electron impact ionisation with selective monitoring of masses 327-330 (Tserng et al., 1984). Measured isotopomer distributions were corrected for natural ^{13}C enrichment at all masses as described previously (Fernandez et al., 1996), using software provided by Dr. Henri Brunengraber (Western Reserve University, Cleveland, OH).

I carried out the measurements for cortisol. Measurements of insulin, C-peptide, growth hormone glucose, urea and electrolytes, HbA_1c, triglycerides, total cholesterol and HDL cholesterol were carried out by the local biochemistry laboratory under the supervision of Mrs Susan Walker. Carbenoxolone measurements were performed by Jill Campbell. Glucagon and free fatty acids were carried out in the biochemistry laboratory at the Newcastle Royal Infirmary under the supervision of Dr Mark Walker. Mrs Wendy Barron and Dr Olav Rooyackers analysed the glucoses and lactates.

Calculation of glucose kinetic parameters

Rates of glucose appearance (R_a) and peripheral glucose disposal (R_d) were calculated from steady state enrichment of the plasma glucose pool with $^{13}C_6$-glucose, using mean data obtained in the basal state (07:00-07:30h), during hyperinsulinaemia (10:00-10:30h) and with the addition of hyperglucagonaemia (12:00-12:30h). All enrichments during these periods were confirmed as steady state by regression coefficients for seven measurements against time not significantly different from zero. Glucose and lactate enrichments achieved in plateau were similar to those reported by Tayek and Katz (Tayek & Katz, 1996). The glucose production rate was calculated by subtracting the glucose infusion rate from R_a.

Gluconeogenesis rates were calculated at the same intervals from the enrichment of the lactate pool with $^{13}C_3/^{13}C_6$-lactate, according to the steady state formulas described by Tayek and Katz (Tayek & Katz, 1996). Glycogenolysis was calculated as (glucose production rate) – (gluconeogenesis rate).

Statistics

Data are expressed as means ± SE. Effects of carbenoxolone within groups were examined by paired Student's t tests. Differences between patient groups were tested by unpaired Student's t tests.

Results

Baseline characteristics

DM and control men were well-matched for age (59±3 vs 58± 3 y, DM vs controls, p=0.94), body mass index (29.2±1.3 vs 29.1±0.9, p=0.94) and waist/hip circumference ratio (0.95±0.01 vs 0.92±0.03, p=0.43). Glycaemic control was excellent in all DM patients, so that HbA_{1C} was only marginally higher than in controls (6.8±0.4 vs 6.0±0.1%, p=0.06). HDL cholesterol was lower in DM patients (Table 4.1).

Effects of carbenoxolone on blood pressure, plasma electrolytes and lipids

Tablet count and plasma carbenoxolone levels confirmed good compliance with study medication (Table 4.1). Carbenoxolone levels tended to be higher in DM than controls (p=0.09). Carbenoxolone raised blood pressure and lowed plasma potassium in both groups as expected, although the effect on plasma potassium was only statistically significant in the DM patients. In contrast, in the control group carbenoxolone decreased fasting plasma cholesterol and tended to increase HDL cholesterol; these effects were not observed in DM patients.

Effects of carbenoxolone on glucose kinetic parameters

Technical success of clamps

The 'technical success' of the clamps is shown in Figure 4.4. Plasma glucose concentrations were maintained similarly close to 5.0 mM throughout the study in

	Controls		Diabetic patients	
	Placebo	Carbenoxolone	Placebo	Carbenoxolone
Weight (kg)	89.5 ± 5.9	89.9 ± 5.7	83.0 ± 5.8	84.2 ± 5.6
Systolic blood pressure (mmHg)	132 ± 8	149 ± 6**	130 ± 6	148 ± 6**
Diastolic blood pressure (mmHg)	79 ± 4	84 ± 4*	78 ± 5	84 ± 6*
Plasma potassium (mM)	4.38 ± 0.08	4.12 ± 0.14	4.30 ± 0.10	4.07 ± 0.12*
Plasma total cholesterol (mM)	5.25 ± 0.34	4.78 ± 0.40**	4.28 ± 0.70	4.33 ± 0.33
HDL cholesterol (mM)	1.28 ± 0.15	1.42 ± 0.15	0.89 ± 0.07@	0.95 ± 0.09@
Plasma triglycerides (mM)	1.75 ± 0.62	1.16 ± 0.37	1.27 ± 0.37	1.00 ± 0.21
Plasma carbenoloxone (mg/l)	all <10	46.8 ± 11.8**	all <10	88.3 ± 18.5**

Table 4.1. Effect of carbenoloxone on clinical characteristics and biochemistry.

Data are mean ± SE

*p<0.05 and **p<0.01 in paired Student's *t* test vs placebo
@P<0.05 IN Student's *t* test vs control group

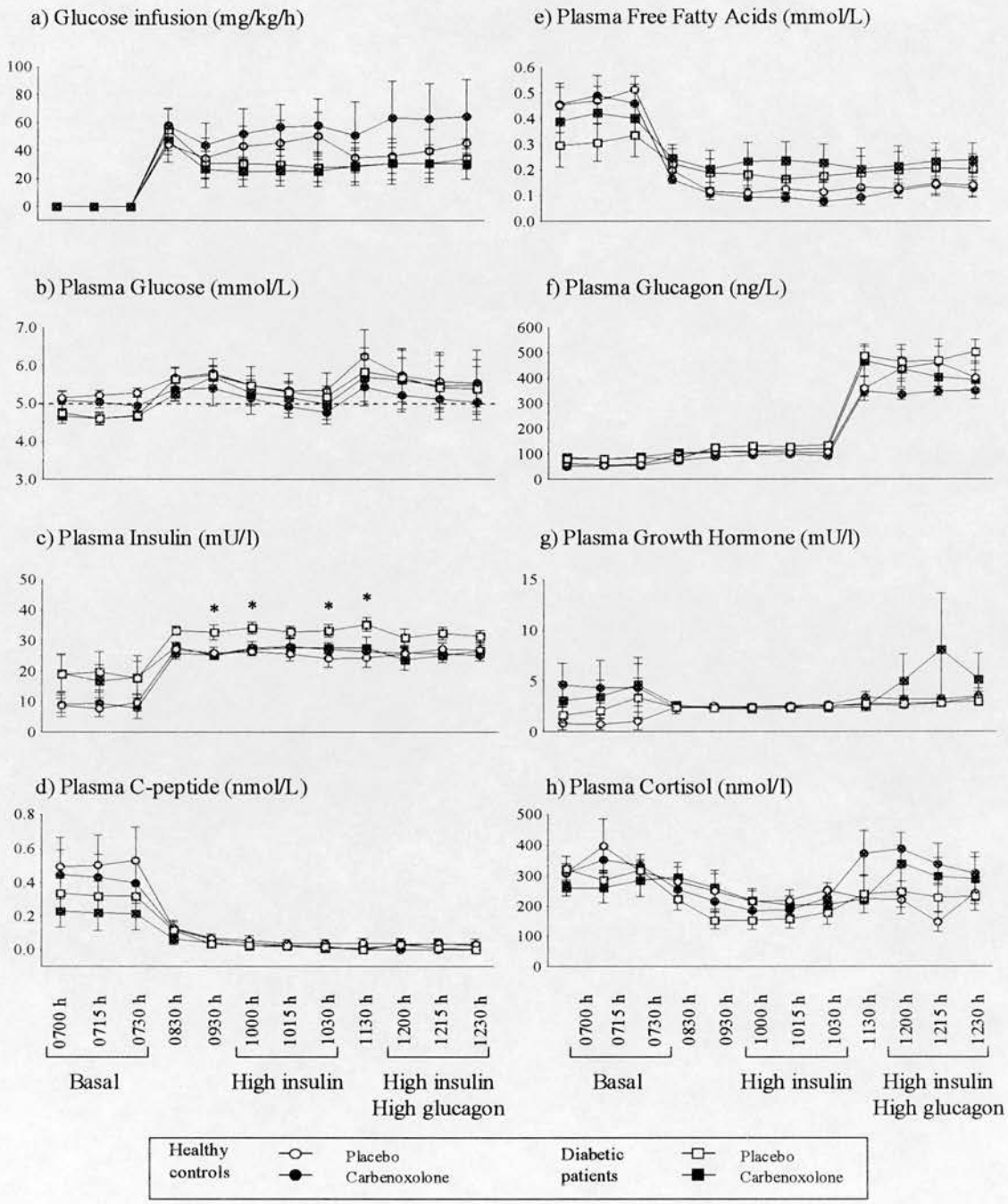

Figure 4.4. Measurement during clamp studies

Data are mean ± SEM. For clarity only limited time points are shown for each measurement. ○ and ● Healthy controls; □ and ■ patients with diabetes, ○ and □, after placebo; ● and ■, after carbenoxolone. *, $P<0.05$ vs. all the other groups (by paired *t* test vs. controls and by paired *t* test vs. DM group during carbenoxolone treatment).

both groups with and without carbenoxolone (Figure 4.4b). To achieve this, insulin was infused at low doses overnight in 5 of the DM patients and 3 of the control subjects (controls after placebo 0.3 ± 0.1 units/h, controls after carbenoxolone 0.4±0.2 units/h, DM after placebo 1.0 ± 0.6 units/h, DM after carbenoxolone 1.3±0.3 units/h). The resulting plasma insulin levels at 07:00-07:30 h tended to be higher in DM patients, irrespective of carbenoxolone therapy (Figure 4.4c). Thereafter, the anticipated degree of hyperinsulinaemia was achieved by infusion of 0.4 mU/kg/min insulin, but resulting insulin concentrations were higher in DM subjects after placebo than in other groups. The incremental rise in insulin concentrations was similar in each group. C-peptide was similar at baseline and suppressed in all subjects during hyperinsulinaemia (Figure 4.4d). Growth hormone was similar at baseline and 'clamped' successfully in all participants, except one DM patient after carbenoxolone whose growth hormone rose to >15 mU/l from 12:00-12:30 h (Figure 4.4g). Plasma glucagon levels were not different at baseline, and were 'clamped' as intended to physiological levels by infusion of 1.5 ng/kg/min and high levels by infusion of 6.0 ng/kg/min (Figure 4.4f). Plasma cortisol followed the normal diurnal rhythm in all groups (Figure 4.4h).

Effect on insulin sensitivity

Glucose was infused at variable rate during hyperinsulinaemia. The rate of glucose infusion plateaued to similar rates in all groups within one hour (Figure 4.4a). There was a non-significant trend for higher absolute infusion rates in healthy controls after carbenoxolone. Free fatty acid levels were not different at baseline and were suppressed similarly during hyperinsulinaemia (Figure 4.4e). Total glucose disposal (R_d)(Figure 4.5a) was stimulated by insulin and rose further with the addition of hyperglucagonaemia, especially in control subjects, but was unaffected by carbenoxolone in either group.

Effect on glucose production, glucoenogenesis and glycogenolysis

Glucose production rates were not different at baseline, suppressed during hyperinsulinaemia, and stimulated during hyperglucagonaemia (Figure 4.5b). These changes in glucose production during the clamp were associated with the expected

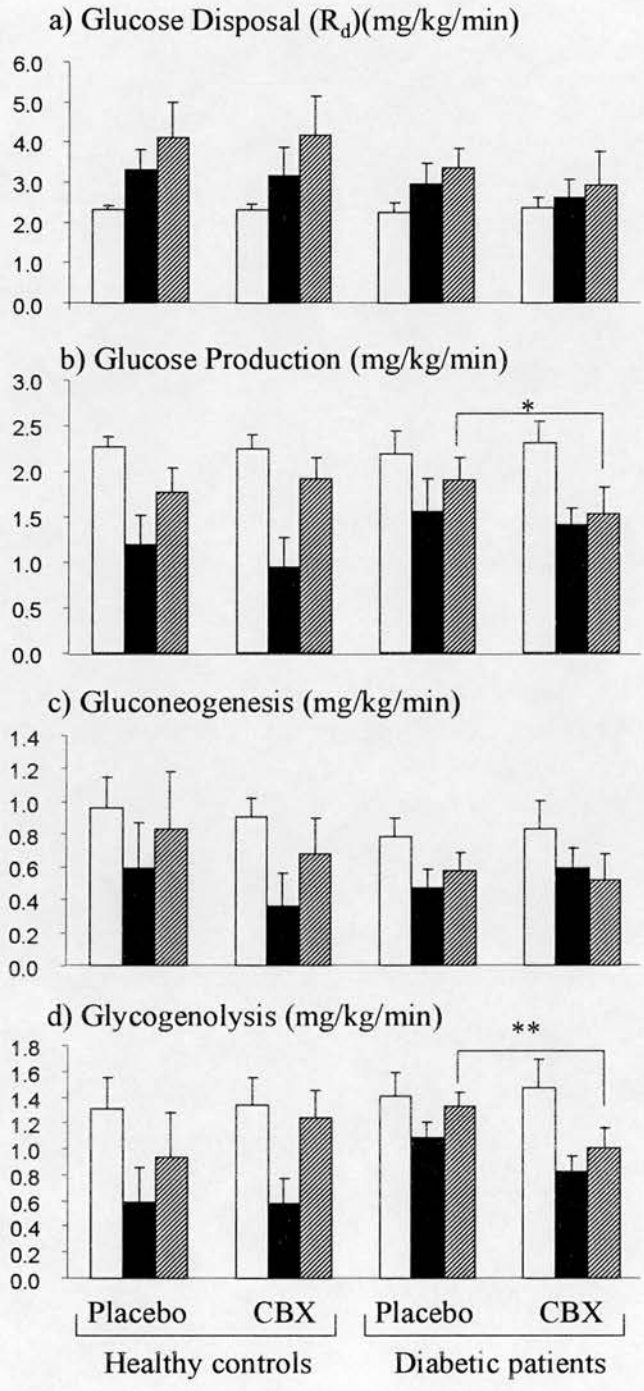

Figure 4.5. Kinetic parameters derived from [$^{13}C_6$] glucose tracer measurements.

Data are mean ± SEM. □, Basal measurements from 07:00-07:30h; ■, measurements during the hyperinsulinemia from 10:00-10:30h, □, measurements during hyperinsulinemia and hyperglucagonemia from 12:00-12:30h. *, $P<0.05$, **, $P<0.01$ (between the groups indicated by paired *t* test).

changes in gluconeogenesis (Figure 4.5c) and glycogenolysis (Figure 4.5d), both of which were suppressed by hyperinsulinaemia and stimulated by hyperglucagonaemia. Carbenoxolone prevented the increase in glucose production rate during hyperglucagonaemia in DM subjects only. This was attributable to reduced glycogenolysis with no significant difference in gluconeogenesis.

Effect of different carbenoxolone concentrations

The influences of inter-individual variations in plasma carbenoxolone concentrations was investigated for each of the variables that were significantly different between carbenoxolone and placebo phases in either group. In Pearsons simple correlations, plasma levels of carbenoxolone were not significantly associated with the difference between measurements during carbenoxolone and placebo phases. Multiple regression was employed to explore whether differences in the effects of carbenoxolone between DM and controls could be accounted for by differences in plasma carbenoxolone levels. Explanatory variables were plasma carbenoxolone concentrations and diagnosis (DM or control, coded as 0 and 1). These models did not show any independent effect of carbenoxolone concentrations.

Discussion

Previous research in mice, rats and healthy humans suggests that inhibition of 11β-HSD1 lowers intrahepatic glucocorticoid concentrations and thereby reduces hepatic glucose production and enhances lipid catabolism (Seckl & Walker, 2001). In addition, more recent evidence suggests that inhibition of 11β-HSD1 in adipose tissue will increase peripheral glucose uptake and suppress lipolysis (Masuzaki et al., 2001). This report extends previous studies using the non-selective 11β-HSD inhibitor, carbenoxolone (Monder et al., 1989) (Stewart et al., 1990) (Walker et al., 1994). We examined in detail its site of action on glucose metabolism in healthy men, and tested its effects in patients with T2DM, a patient group which might benefit from therapeutic use of selective 11β-HSD1 inhibitors in future. We employed a detailed protocol to control for variables which are sometimes neglected during euglycaemic clamp studies, including overnight preparation of subjects to avoid effects of baseline hyperglycaemia (Neilsen et al., 1998) (Vaag et al., 1995),

successful 'clamping' of growth hormone and glucagon levels, and stable isotope tracer measurement of gluconeogenesis. We have shown that one week of carbenoxolone administration decreased glucagon-stimulated glucose production and glycogenolysis in patients with T2DM but not healthy subjects, and decreased total cholesterol in healthy controls but not patients with T2DM. Carbenoxolone had no effects on gluconeogenesis, peripheral glucose uptake or insulin-mediated suppression of plasma free fatty acids in either the healthy control subjects or the patients with T2DM. These observations reinforce the potential value of 11β-HSD1 inhibitors in enhancing hepatic insulin sensitivity and lipid catabolism.

An important consideration in designing this study to test the utility of 11β-HSD1 inhibition in metabolic disease was raised by observations that there are tissue-specific alterations in enzyme activity in obesity. Thus, 11β-HSD1 is increased in adipose tissue but decreased in liver in obesity (Paulmyer-Lacroix et al., 2002) (Rask et al., 2001) (Rask et al., 2003) (Stewart et al., 1999) (Livingstone et al., 1999). In contrast, lean patients with type 2 diabetes do not have marked dysregulation of either adipose or hepatic 11β-HSD1 (Kerstens et al., 2000) (Andrews et al., 2002). In order to avoid the potential confounding effects of obesity, and to exclude any unknown effects of oral hypoglycaemic or antihypertensive agents, we selected non-obese normotensive patients with T2DM controlled by dietary therapy alone. The result was that patients in this study were not typical of T2DM. Indeed, they had near-normal blood glucose and HbA_{1C} levels, a small requirement for overnight insulin infusion to obtain fasting euglycaemia, and only minor differences in plasma lipids (Table 4.1). Following overnight euglycaemia with insulin infusion as required, glucose production, free fatty acids, and glucagon levels were not elevated in these patients with T2DM, and glucose disposal was not measurably impaired. Nonetheless, effects of carbenoxolone differed between healthy controls and patients with T2DM; an effect on cholesterol was only evident in healthy controls, and measurable effects on glucose production were only evident in patients with T2DM.

In a previous study, it has been shown that the same regime of carbenoxolone administration to healthy men resulted in enhanced insulin sensitivity, as measured

by increased glucose infusion rate during a hyperglycaemic clamp (Walker et al., 1994). A key difference, however, is that the previous study was performed with a higher insulin infusion rate, achieved higher insulin concentrations (~70 mU/l compared with ~30 mU/l here), and was designed to examine effects on insulin-stimulated glucose uptake rather than glucose production (Rizza et al., 1981). Also, growth hormone and glucagon levels were not clamped in the previous study and the participants were younger. In the current study, there was a trend for a similar magnitude of increase in glucose infusion rate in healthy controls (means differed by ~7% previously, and by ~17% here; see Figure 4.4a), but this did not reach statistical significance. Glucose production was marginally, but not significantly, lower in healthy men after carbenoxolone in the current study. This contrasts with the statistically significant effects of carbenoxolone on glucose kinetics in patients with T2DM.

For unknown reasons, plasma carbenoxolone levels tended to be higher in the patients with T2DM than in controls (see table 4.1), so that the effects of carbenoxolone could have been under estimated in control subjects. Single measurements of plasma carbenoxolone concentrations were included in this study principally as a qualitative assessment of compliance. A more detailed pharmacokinetic study would be required to confirm that this difference did not occur by chance. Importantly, however, in multiple regression analysis the variations in carbenoxolone concentrations between individuals did not account for the different effects of carbenoxolone in patients with T2DM and healthy controls. Insulin concentrations during the clamp studies were also higher in patients with T2DM during placebo therapy than in all other groups (see figure 4.4c), which may lead to underestimation of the effects of carbenoxolone in patients with T2DM. Against this background, it is unclear whether quantitative or qualitative differences explain the discrepancies between effects of carbenoxolone in health and diabetes, although, we suspect the former.

This is the first report of the effects of carbenoxolone, or any 11β-HSD inhibitor, in patients with T2DM. It shows that carbenoxolone affects glucose production, as

inferred indirectly from a previous report {Walker et al., 1994}, but the mechanism of the effect was not expected. In 11β-HSD1 knockout mice a key feature is impaired up-regulation of gluconeogenic enzymes, such as PEPCK, on fasting (Kotelevtsev et al., 1997). Glucocorticoids are known to oppose the effect of insulin in regulating expression of gluconeogenic enzymes (Andrews & Walker, 1999). However, carbenoxolone did not alter gluconeogenesis after overnight fast, during hyperinsulinaemia, or during hyperglucagonaemia. One consideration in this paradox is that the contribution of the kidney to gluconeogenesis in man remains unquantified. By inhibiting inactivation of cortisol by 11β-HSD2 in kidney (Stewart et al., 1990), carbenoxolone increases intrarenal cortisol concentrations, which might enhance renal gluconeogenesis in compensation. To resolve this will require studies either with selective 11β-HSD1 inhibitors or with cannulation of the hepatic and/or renal veins. The kidney is not, however, a major site of glycogen storage. Glucocorticoids have complex effects on glycogenic and glycogenolytic enzymes, which predict increased turnover and amplification of the effect of other signals (Andrews & Walker, 1999) (Rooney et al., 1994). Thus, the observation that carbenoxolone attenuated net glucagon-induced glycogenolysis is consistent with lowering of intrahepatic cortisol concentrations.

A more recently recognised consequence of changes in intra-hepatic glucocorticoid concentrations is the effect on lipid metabolism (Morton et al., 2001). The effects of carbenoxolone in the liver are the most likely explanation for the decrease in total cholesterol observed in healthy controls. In 11β-HSD1 knockout mice, hepatic lipid catabolism is markedly increased while synthesis is relatively normal, resulting in elevated HDL-cholesterol and reduced total cholesterol (Morton et al., 2001). However, the importance of enhanced lipid catabolism in man, and comparison of effects in healthy controls and patients with T2DM, needs to be re-assessed with a longer duration of carbenoxolone administration, since plasma lipids take several weeks to re-equilibrate following the introduction of conventional lipid-lowering therapy.

Liquorice derivatives, such as carbenoxolone and glycyrrhetinic acid, are potent inhibitors of both isozymes of 11β-HSD *in vitro* and in cell culture (Monder et al., 1989) (Rajan et al., 1996) (Yang, et al., 1997). However, *in vivo* they have inconsistent effects, probably because of pharmacokinetic differences in access to tissues. Thus, carbenoxolone, but not glycyrrhetinic acid, inhibits hepatic 11β-HSD1 *in vivo* in man, as judged by impaired generation of cortisol after an oral dose of cortisone but has no effect on adipose 11β-HSD1 activity (TC Sandeep et al., unpublished observations) (Livingstone & Walker, 2003). In animals, *in vivo* inhibition of 11β-HSDs with carbenoxolone in other tissues is also inconsistent, for example varying between different regions of CNS (Ajilore & Sapolsky, 1999) (Jellinck et al., 1993). Indeed, in Zucker obese rats, *in vivo* administration of carbenoxolone inhibits 11β-HSD1 in liver but not in adipose tissue (Livingstone & Walker, 2003). In this study, relatively modest hyperinsulinaemia was employed in order to approximate the ED_{50} for suppression of hepatic glucose production (Rizza et al., 1981). For these reasons, the positive effects of carbenoxolone on hepatic carbohydrate and lipid metabolism, but lack of effect on peripheral glucose uptake in both the current and previous study (Walker et al., 1994), does not allow the conclusion that inhibition of 11β-HSD1 in extrahepatic tissues, notably adipose tissue, would not be beneficial. However, the acute effects of carbenoxolone on hepatic insulin sensitivity were of modest magnitude. For more substantial effects on glucose tolerance and glycaemic control in patients with T2DM, it appears likely that 11β-HSD1 inhibitors will be required to inhibit glucocorticoid regeneration in adipose tissue as well as the liver. Reducing cortisol action in adipose tissue may then provide an increase in peripheral glucose disposal in addition to the reduced glucose production observed with carbenoxolone.

Conclusion

In summary, these studies with a non-selective 11β-HSD inhibitor illustrate the potential value of inhibition of 11β-HSD1 in lean hyperglycaemic patients. It may well be that greater benefits will be obtained in obese patients and in patients with dyslipidaemia.

CHAPTER 5

CONCLUSIONS

Overview

Type 2 diabetes (T2DM) is the most common clinical form of diabetes, accounting for 90% of all cases. In the UK about 1.4 million people are known to suffer with this disease, however, this figure is predicted to triple over the next 10 years Unlike Type 1 diabetes, the pathogenesis of T2DM remains only partly understood. Both insulin resistance and impaired insulin secretion are required for the development of disease but what order these abnormalities occur in and what causes them remains unknown.

Recent research has suggested that it is unlikely that one primary cause for T2DM will be found and that both genetic and environmental factors will contribute to the development of this disease. Furthermore the finding that impaired glucose intolerance, insulin resistance, essential hypertension, obesity and coronary heart disease occur together more commonly than would be expected by chance, suggests that many of the factors found to play a part in the pathogenesis of T2DM will also be important in the development of these diseases (Reavan, 1988).

For many years it has been known that cortisol antagonises the action of insulin, i.e. induces a state of insulin resistance. In the liver it increases glucose production, in the periphery it impairs insulin-dependent glucose uptake and in the brain it stimulates appetite (Rizza et al., 1982) (Holmang & Bjorntorp, 1992) (Stubbs & York, 1991). In addition to these effects on insulin sensitivity, cortisol also inhibits insulin secretion from the pancreatic β-cells (Delaunay et al., 1997) (Ling et al., 1998). Furthermore it is common knowledge that conditions characterised by excessive cortisol concentrations, such as Cushing's disease, are associated with hypertension, obesity, coronary artery disease and glucose intolerance. For this reason cortisol was one of the first hormones to be implicated in the pathogenesis of T2DM and its associated diseases.

However, early studies found that crude measures of cortisol secretion were normal in these individuals and so little advanced further in this field. In the late 1980s two enzymes 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD 1) and 11beta-

hydroxysteroid dehydrogenase type 2 (11β-HSD 2) were cloned (Agarwal et al., 1989) (Agarwal et al., 1994). These control the conversion of cortisol to cortisone and vice versa in the tissues meaning that tissues may be exposed to a relative excess of cortisol without any increase in cortisol secretion or plasma cortisol concentrations (Seckl & Walker, 2001).

Cortisol activity and the metabolic syndrome

Individuals with hypertension were one of the first groups to be studied. They were noted to exhibit normal cortisol secretion, but have reduced inactivation of cortisol by 11β-HSD 2 and enhanced tissue sensitivity to cortisol (Soro et al., 1995) (Walker et al., 1992) (Walker et al., 1996). Abnormalities were also found in obese individuals with an increase in 24 hour cortisol secretion in spite of enhanced feedback sensitivity. Again, plasma cortisol concentrations were not elevated, perhaps because of an increase in peripheral clearance of cortisol (Strain et al., 1982) (Ljung et al., 1996) (Andrew et al., 1998) (Rask et al., 2001). These findings led people to suggest that abnormalities in cortisol could be one of the common factors involved in the pathogenesis of diabetes and its associated conditions (Bjorntorp et al., 1999).

Cortisol activity in patients with type 2 diabetes

Against this background, I decided to investigate whether abnormalities in cortisol secretion, metabolism or sensitivity exist in patients with type 2 diabetes or glucose intolerance. Previous studies in this area had been conducted in heterogeneous groups with type 1 and type 2 diabetes (Mortimore et al., 1956) (Lentle & Thomas, 1964) (Huther & Scholz, 1970) (Tsigos et al., 1993) (Hudson et al., 1984) (Kaye et al., 1992), or had inadequately controlled for confounding factors of sex, co-existing obesity, hypertension, poor glycaemic control and diabetic complications making interpretation difficult (Couch, 1992) (Dullaart et al., 1995) (Roy et al., 1993) (Stewart et al., 1990) (Lentle & Thomas, 1964) (Walker et al., 1998).

In this thesis I have examined cortisol secretion, metabolism and sensitivity in non-obese, normotensive, diet-controlled male patients with type 2 diabetes mellitus or

impaired glucose tolerance. These individuals did exhibit abnormalities in cortisol activity. Specifically they showed:

1) Normal cortisol secretion and circulating levels in the face of enhanced negative feedback sensitivity
2) Enhanced *in vivo* peripheral tissue sensitivity to glucocorticoids
3) Impaired hepatic 11β-HSD 1 activity but normal adipose 11β-HSD 1 activity, suggesting tissue-specific alterations in 11β-HSD 1 activity.
4) Increased relative excretion of A-ring reduced metabolites of cortisol.

These findings suggest that isolated hyperglycaemia is associated with some, but not all, of the changes in cortisol metabolism and action which have been observed in subjects with hypertension and obesity, and adds further weight to the hypothesis that abnormalities in cortisol action may be important in the pathogenesis of T2DM and its associated conditions.

Cortisol secretion

The finding of normal 24h secretion in the face of this enhanced feedback sensitivity suggests that another factor is driving cortisol secretion. This could be increased metabolism by 5α-reductase, enhanced by some yet to be defined mechanism which is associated with relative insulin deficiency and/or hyperglycaemia or by inappropriate central drive to the HPA axis which is consistent with the observation that habituation of cortisol in response to repeated sampling is impaired in hyperglycaemic men (Tsigos et al., 1993) (Reynolds et al., 2001). Further research is required in this area to try and determine which of these it is.

11β-HSDs activity

In obese humans 11β-HSD 1 is decreased in liver (Stewart et al., 1999) but increased in adipose tissue (Rask et al., 2002) (Paulmyer-Lacroix et al., 2002) (Rask et al., 2001). Here, in non-obese hyperglycaemic men, hepatic first pass conversion of cortisone to cortisol was impaired, albeit to a lesser extent than in obese subjects (Rask et al., 2001) (Stewart et al., 1999). However, 11β-HSD 1 activity in gluteal

adipose tissue was normal. These data suggest that hepatic dysregulation is related to insulin action while adipose dysregulation is determined by some other factor associated with obesity, or indeed may be a primary mechanism in obesity (Bujalska et al., 1997) (Masuzaki et al., 2001). There are two possible explanations for these tissue specific alterations in β-HSD 1 activity; 1) that local growth factors such as TNFα, interleukin1β and leptin regulate 11β-HSD 1 expression as has been shown *in vitro* (Tomlinson et al., 2001); or 2) that there is a different type (polymorphism) of 11β-HSD 1 in each tissue.

Vascular sensitivity

The finding that dermal vascular sensitivity to beclomethasone dipropionate is increased in patients with type 2 diabetes mellitus or impaired glucose tolerance suggests that glucocorticoid receptors are more readily activated in dermal vessels. This could be explained by increased access of hormones to the intracellular glucocorticoid receptors (GR), increased GR number, affinity or signalling, or altered sensitivity of the dermal blood vessels to the products of the glucocorticoid target genes.

Previous studies have demonstrated that there is little difference in the *in vitro* vasodilation or vasocontrictive responses between healthy control subjects and patients with T2DM (Andrews et al., 1999). Furthermore, in this thesis, no differences in distribution or concentration of GR mRNA between patients with hyperglycaemia and normal healthy controls was found. This suggests that either alteration in the relative activity of the 11β-HSDs or differences in polymorphisms of the GR will explain the increase in the dermal vascular sensitivity to beclomethasone dipropionate which is seen in patients with type 2 diabetes mellitus or impaired glucose tolerance.

Although data presented in this thesis add to the body of evidence that suggests that abnormalities in cortisol activity are important in the pathogenesis of T2DM and its related diseases, it by no means proves this. One could argue that these abnormalities are present as a result of the diseases rather than the cause of them. In

order for pathogenesis to be proven, these abnormalities must be shown to occur before the disease develops and must also be shown to be altered by those factors which are known to protect against disease states, in the case of T2DM this being diet and exercise. Thus longitudinal and interventional studies will be needed.

Novel treatments for type 2 diabetes

As I mentioned earlier, much of the action of cortisol depends on it opposing the action of insulin, namely inducing a state of insulin resistance. From this it would seem that drugs designed to lower plasma cortisol concentrations would be ideal for treating patients with T2DM. Unfortunately though, cortisol also plays a key role in regulating growth, salt and water retention, maintaining blood pressure and enabling the body to respond to stressful events meaning that lowering plasma cortisol concentrations could have many serious side-effects. If a way could be found to lower tissue concentrations of cortisol in the liver, fat and muscle without altering plasma cortisol levels this could be a safe and effective drug for treating T2DM.

Confirmation in animal studies that alteration in 11β-HSD1 expression did not unduly affect plasma cortisol concentrations {Kotelevtsev, Holmes, et al. 1997 1164 /id}{Masuzaki, Paterson, et al. 2001 1573 /id} suggested that pharmacological inhibition of 11β-HSD1 might be an exciting potential therapy in T2DM. At present, only relatively non-selective inhibitors of 11β-HSD1 are available for human use. The principal active constituent of confectionary liquorice, glycyrrhetinic acid, and its hemisuccinate derivative carbenoxolone, are potent inhibitors of both 11β-HSD1 and 11β-HSD type 2 {Monder, Stewart, et al. 1989 165 /id}. Inhibition of 11β-HSD2 with liquorice derivatives results in cortisol-dependent mineralocorticoid excess with hypertension and hypokalaemic alkalosis {Stewart, Valentino, et al. 1987 27 /id}{Stewart, Wallace, et al. 1990 12 /id}. However, in addition, carbenoxolone inhibits regeneration of cortisol from cortisone by 11β-HSD1 in liver {Stewart, Wallace, et al. 1990 12 /id}{Andrew, Smith, et al. 2002 1580 /id} suggesting that it might well improve insulin sensitivity.

A previous study in healthy men had found that carbenoxolone improved insulin sensitivity, as measured by an increase in glucose infusion rate during a euglycaemic hyperinsulinaemic clamp {Walker, Connacher, et al. 1994 760 /id}. In this study there was no effect on peripheral glucose uptake, measured by arteriovenous sampling across the forearm, so it was inferred that carbenoxolone was working by lowering intrahepatic cortisol concentrations with a resulting improvement of insulin dependent suppression of hepatic glucose production.

Effect of carbenoxolone in patients with type 2 diabetes

In the second study described in this thesis I characterised the mechanism of action of carbenoxolone on insulin sensitivity in healthy men, and quantified its effects for the first time in patients with T2DM. In doing so I found that carbenoxolone decreased glucagon-stimulated glucose production and glycogenolysis in patients with T2DM but not healthy subjects, and decreased total cholesterol in healthy but not patients with T2DM. Carbenoxolone also had no effects on gluconeogenesis or peripheral glucose uptake in either the healthy control subjects or the patients with T2DM. These observations reinforce the potential value of 11β-HSD1 inhibitors in enhancing hepatic insulin sensitivity and lipid catabolism.

The fact that carbenoxolone did not alter gluconeogenesis came as somewhat of a surprise, especially as animal studies has suggested that glucocorticoids are potent regulators of the expression of gluconeogenic enzymes (Andrews & Walker, 1999) and that 11β-HSD1 knockout animals show reduced PEPCK and other gluconeogenic enzymes (Kotelvtsev et al., 1997). One consideration in this paradox is that the contribution of the kidney to gluconeogenesis in man remains unquantified. By inhibiting inactivation of cortisol by 11β-HSD2 in kidney (Stewart et al., 1990), carbenoxolone increases intrarenal cortisol concentrations, which might enhance renal gluconeogenesis in compensation. To resolve this will require studies either with selective 11β-HSD1 inhibitors or with cannulation of hepatic and/or renal vein.

The findings that carbenoxolone attenuated net glucagon-induced glycogenolysis in patients with T2DM and that it decreased total cholesterol in healthy controls is consistent with lowering of intrahepatic cortisol concentrations. In 11β-HSD1 knockout mice, animals that have low hepatic concentrations of cortisol, hepatic lipid catabolism is markedly increased while synthesis is relatively normal, resulting in elevated HDL-cholesterol and reduced total cholesterol (Morton et al., 2001). Furthermore glucocorticoids are known to upregulate glycogenolytic enzymes (Andrews & Walker, 1999) (Rooney et al., 1994).

In this study, relatively modest hyperinsulinaemia was employed in order to approximate the ED_{50} for suppression of hepatic glucose production (Rizza et al., 1981). For these reasons, the positive effects of carbenoxolone on hepatic carbohydrate and lipid metabolism, but lack of effect on peripheral glucose uptake, does not allow the conclusion that inhibition of 11β-HSD1 in extrahepatic tissues, notably adipose tissue, would not be beneficial. However, the acute effects of carbenoxolone on hepatic insulin sensitivity were of modest magnitude. For more substantial effects on glucose tolerance and glycaemic control in patients with T2DM, it appears likely that 11β-HSD1 inhibitors will be required to inhibit glucocorticoid regeneration in adipose tissue as well as the liver.

Potential treatments for the future

The liver is central in glucose homeostasis and has a major causative role in T2DM with as much as 90% of hepatic glucose output due to increased gluconeogenesis. As was demonstrated in the introduction of this thesis a major site of action of glucocorticoids is on the expression and activation of enzymes involved in gluconeogenesis. It thus seems reasonable that if drugs could be targeted at reducing cortisol concentrations in the liver then they could have considerable influence on blood glucose concentrations. This has been highlighted in a recent study in which a selective inhibitor of 11β-HSD 1 decreased blood glucose concentrations in hyperglycaemic mice (Alberts et al., 2002). Present drug treatment has little impact on hepatic glucose output suggesting that drugs targeted here could provide an additional tool for the treatment of T2DM.

This thesis has demonstrated that local inhibition of cortisol production by inhibition of 11β-HSD 1 could be a novel target for the treatment of T2DM. If drugs to inhibit 11β-HSD 1 are to be designed they must be specific for this isoenzyme as any inhibition of 11β-HSD 2 will result in intolerable side effects of hypertension, salt retention and hyperkalemia. Furthermore these drugs must not cross react with other enzymes or receptors. If these criteria can be met then an effective new treatment for diabetes is possible.

In summary this thesis has demonstrated that abnormalities in cortisol activity are present in patients with T2DM and that inhibition of 11β-HSD1 might be a novel therapeutic target for the treatment of T2DM. Future research is now needed to clarify whether these abnormalities in cortisol activity result from or are a cause of T2DM.

CHAPTER 6
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CHAPTER 7

APPENDICES

PUBLICATIONS ARISING FROM THIS THESIS

Andrews RC, Rooyackers O, Walker BR. Effect of the 11ß-hydroxysteroid dehydrogense inhibitor carbenoxolone on insulin sensitivity in men with Type 2 diabetes. JCEM: 88; 285-291, 2003

Andrews RC, Herlihy O, Livingstone DEW, Andrew R, Walker BR. Abnormal cortisol metabolism and tissue sensitivity to cortisol in patients with glucose intolerance. JCEM: 87; 5587-5593, 2002

McIntyre CA, Buckley CH, Jones GC, Sandeep TC, Andrews RC, Elliott, AI, Gray, GA, Williams BC, Mcknight JA, Walker BR, Hadoke PWF. Endothelium-derived hyperpolarizing factor and potassium use different mechanisms to induce relaxation of human subcutaneous resistant arteries. British Journal of Pharmacology:133;902-908, 2001.

Andrews RC and Walker BR. Glucocorticoids and insulin resistance: old hormones, new targets. Clinical Science:96;513-523, 1999

Finken MJJ, Andrews RC, Andrew R, Walker BR. Cortisol metabolism in healthy young adults: sexual dimorphism in activities of A-ring reductases, but not 11β-Hydroxysteriod dehydrogenases. JCEM:84;3316-3321, 1999.

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Effects of the 11β-Hydroxysteroid Dehydrogenase Inhibitor Carbenoxolone on Insulin Sensitivity in Men with Type 2 Diabetes

ROBERT C. ANDREWS, OLAV ROOYACKERS, AND BRIAN R. WALKER

University of Edinburgh, Endocrinology Unit, Department of Medical Sciences, Western General Hospital, Edinburgh, United Kingdom EH4 2XU

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) regenerates cortisol from inactive cortisone in liver and adipose tissue. Inhibition of 11β-HSD1 offers a novel potential therapy to lower intracellular cortisol concentrations and thereby enhance insulin sensitivity and hepatic lipid catabolism in type 2 diabetes, obesity, and hyperlipidemia. We evaluated this approach using the nonselective 11β-HSD inhibitor, carbenoxolone, in healthy men and lean male patients with type 2 diabetes.

Six diet-controlled nonobese diabetic patients with hemoglobin A_{1c} less than 8%, and six matched controls participated in a double-blind, cross-over comparison of carbenoxolone (100 mg every 8 h, orally, for 7 d) and placebo. They were admitted overnight for infusions of insulin (as required to maintain arterialized plasma glucose of 5.0 mM) and [$^{13}C_6$]glucose. Glucose kinetics were measured in the fasted state from 0700–0730 h, during a 3-h euglycemic hyperinsulinemic clamp (including somatostatin infusion and replacement of physiological GH and glucagon levels), and during a 2-h euglycemic hyperinsulinemic clamp with a 4-fold increase in glucagon levels. Data are the mean ± SEM.

Carbenoxolone had the expected effects of raising blood pressure and lowering plasma potassium. Carbenoxolone reduced total cholesterol in healthy subjects (5.25 ± 0.34 *vs.* 4.78 ± 0.40 mM; $P < 0.01$), but had no effect on other serum lipids or on cholesterol in diabetic patients. Carbenoxolone did not affect the rate of glucose disposal or the suppression of free fatty acids during hyperinsulinemia. However, carbenoxolone reduced the glucose production rate during hyperglucagonemia in diabetic patients (1.90 ± 0.2 *vs.* 1.53 ± 0.3 mg/kg·min; $P < 0.05$). This was attributable to reduced glycogenolysis (1.31 ± 0.2 *vs.* 1.01 ± 0.2 mg/kg·min; $P < 0.005$) rather than altered gluconeogenesis.

These observations reinforce the potential metabolic benefits of inhibiting 11β-HSD1 in the liver of patients with type 2 diabetes. Further studies in obesity and hyperlipidemia are now warranted. However, clinically useful therapeutic effects will probably require selective 11β-HSD1 inhibitors that lower intraadipose cortisol levels and enhance peripheral glucose uptake. (*J Clin Endocrinol Metab* 88: 285–291, 2003)

11β-HYDROXYSTEROID dehydrogenase type 1 (11β-HSD1) is an enzyme that regenerates the active glucocorticoid cortisol from its inactive metabolite cortisone (1). Its potential importance in obesity and type 2 diabetes mellitus has been thrown into sharp focus by recent observations in mice. Animals with 11β-HSD1 knockout have normal or marginally increased plasma glucocorticoid levels, but cannot regenerate glucocorticoid within cells in liver and adipose tissue. As a result, they are protected from the insulin resistance, hyperglycemia (2), and weight gain (Morton, N. M., *et al.*, unpublished observations) induced by high fat feeding (3). Similarly, down-regulation of 11β-HSD1 expression after the administration of estradiol to male rats is associated with decreased markers of hepatic gluconeogenesis (4). Conversely, mice with transgenic overexpression of 11β-HSD1 selectively in adipose tissue under the aP2 promoter have increased intraadipose glucocorticoid concentrations despite no change in plasma levels (5). These animals have a dramatic phenotype of central obesity, insulin resistance, and hyperglycemia. Mice with transgenic overexpression selectively in liver under the apolipoprotein E promoter also show insulin resistance and hyperlipidemia (Paterson, J. M., *et al.*, unpublished observations). In idiopathic obesity in man 11β-HSD1 activity is selectively increased in adipose tissue (6–8) to a similar degree as the increase in transgenic overexpressing mice. Thus, increased 11β-HSD1 in adipose tissue may be a key mechanism determining the predisposition to obesity in man in what has been coined Cushing's disease of the omentum (9). Pharmacological inhibition of 11β-HSD1 to lower intracellular cortisol concentrations in liver and adipose tissue, without altering circulating cortisol concentrations or responses to stress, is an exciting potential therapy in type 2 diabetes and obesity.

Relatively nonselective inhibitors of 11β-HSD1 are available for human use. The principal active constituent of confectionary liquorice, glycyrrhetinic acid, and its hemisuccinate derivative, carbenoxolone, are potent inhibitors of both 11β-HSD1 and its isoenzyme, 11β-HSD2 (10). 11β-HSD2 is expressed principally in the distal nephron, where it inactivates cortisol to cortisone and thereby protects mineralocorticoid receptors from cortisol (11, 12). Inhibition of 11β-HSD2 with liquorice derivatives results in cortisol-dependent mineralocorticoid excess with hypertension and hypokalemic alkalosis (13, 14). However, in addition carbenoxolone in-

Abbreviations: DM, Diabetes mellitus; HbA_{1c}, hemoglobin A_{1c}; HDL, high density lipoprotein; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; R_a, rate of glucose appearance; R_d, rate of peripheral glucose disposal.

hibits regeneration of cortisol from cortisone by 11β-HSD1 in liver (14, 15). In a previous study of healthy men we showed that carbenoxolone increased insulin sensitivity, as measured by an increase in glucose infusion rate during euglycemic hyperinsulinemic clamp (16). There was no effect on peripheral glucose uptake, measured by arterio-venous sampling across the forearm, so it was inferred that carbenoxolone lowers intrahepatic cortisol concentrations and thereby prevents insulin-dependent suppression of hepatic glucose production.

In the present study we aimed to characterize the mechanism of action of carbenoxolone on insulin sensitivity in healthy men and quantify its effects for the first time in patients with type 2 diabetes. The selection of patients most likely to respond to 11β-HSD1 inhibition was an important consideration. In obese patients there is tissue-specific dysregulation of 11β-HSD1, resulting in increased regeneration of cortisol in adipose tissue (6–8) but decreased activity in liver (6, 17). In contrast, in lean patients with type 2 diabetes we found a relatively small decrease in hepatic 11β-HSD1 activity and no change in the enzyme in adipose tissue (18). It is not established that carbenoxolone effectively inhibits 11β-HSD1 in adipose tissue, but it does inhibit 11β-HSD1 in liver (Livingstone, D. E. W., *et al.*, unpublished observations) (14). For these reasons we recruited only lean patients with type 2 diabetes in the current study and aimed principally to study the effects of carbenoxolone in the liver.

Subjects and Methods

Participants

We studied six men with type 2 diabetes mellitus (diagnosed <3 yr previously by WHO criteria; DM group) recruited from our clinic and six normal healthy controls recruited by advertisement. Patients were treated with diet alone, without oral hypoglycemic agents or insulin, and were free of retinopathy, nephropathy, and neuropathy at their most recent annual review. Exclusion criteria included body mass index greater than 32 kg/m^2, weight loss greater than 5 kg in the previous 3 months, therapy for any other medical conditions, including dyslipidemia and hypertension, blood pressure greater than 160/90 mm Hg, major psychiatric disorder, abnormal renal or thyroid function on biochemical screening, or glucocorticoid therapy by any route in the previous 3 months. Healthy control men were matched for age, weight, height, body mass index, and blood pressure. Local ethical committee approval and written informed consent were obtained.

Protocol

Participants took carbenoxolone (100 mg every 8 h, orally, for 7 d) or placebo in a double-blind, randomized, cross-over trial with phases separated by at least 3 months of washout. This dose of carbenoxolone has been shown previously to inhibit conversion of cortisone to cortisol in man (14, 15). On d 4 of each phase measurements of weight, blood pressure and plasma electrolytes were made to avoid adverse effects of carbenoxolone (hypokalemia and sodium retention), but no subject had to be withdrawn. On the evening of the seventh day of each phase, participants were admitted to the Clinical Research Facility for clamp studies. Compliance with study medication was monitored by tablet counting and by measuring plasma carbenoxolone levels in samples obtained at 0700 h on the eighth day.

Euglycemic clamp protocol

Participants attended the clinical research facility at 1730 h for a standardized meal. Thereafter their only oral intake was water. Cannulas were placed in an antecubital vein for infusions and retrogradely in a contralateral dorsal hand vein; the hand was kept in a hot box for arterialized blood sampling. The clamp was divided into three phases (Fig. 1).

Phase 1. From 2200–0730 h, an overnight clamp was employed to ensure that controls and DM patients started the hyperinsulinemic clamps with similar plasma glucose concentrations and to measure basal parameters. Blood glucose was measured at least every 15 min, and iv insulin was administered at variable rates, if required, to maintain glucose at 5.0 mM. From 0400 h, [$^{13}C_6$]glucose was infused (at 5 mg/kg·h after priming with 5 mg/kg).

Phase 2. From 0730–1030 h, a hyperinsulinemic, normoglucagonemic, normoglycemic clamp was performed with infusions of insulin (0.4 mU/kg·min), somatostatin (0.25 mg/h), glucagon (1.5 ng/kg·min), GH (3 ng/kg·min), and 20% glucose. The 20% glucose infusion rate was varied to maintain arterialized blood glucose at 5.0 mM.

Phase 3. From 1030–1230 h, a hyperinsulinemic, hyperglucagonemic, normoglycemic clamp was performed by increasing the glucagon infusion rate from 1.5 to 6.0 ng/kg·min while maintaining other infusions.

In addition to frequent samples for bedside blood glucose monitoring, blood samples were obtained as indicated in Fig. 1. Blood was immediately centrifuged, and the plasma was frozen and stored at −80 C until analysis.

Laboratory analyses

Enzyme immunoassays (Eurogenetics Tasah Corp. UK Ltd., Hampton, UK) were used to measure plasma insulin, GH, and C peptide. Electrolytes were measured with a Vitras 950 (Ortho Diagnostics, Raritan, NJ), and glucose was determined on a Cabas Mira Plus (Roche, Mannheim, Germany). Triglycerides, total cholesterol, and high density lipoprotein (HDL) cholesterol were measured using ELISA kits (TG, CHOL, and HDL C-plus, respectively; Roche). Hemoglobin A_{1c} (HbA_{1c}) was measured by ion exchange HPLC (Variant 11, Bio-Rad Laboratories, Inc., Richmond, CA). RIAs were used to measure cortisol (19) and glucagon (20). Free fatty acids were measured by a colorimetric technique (Wako, Neuss, Germany). Carbenoxolone was measured by HPLC with UV detection (at 254 nm) using 18α-glycyrrhetinic acid as an internal standard.

Enrichment of glucose isotopomers was analyzed as its acetylated di-*O*-isopropylidene derivative (21) using a gas chromatograph quadrupole mass spectrometer (Voyager, Thermoquest, Manchester, UK). Electron impact ionization was used with selective monitoring of masses 287–293. Enrichment of lactate isotopomers was analyzed as its propylamideheptafluorobutyric acid using electron impact ionization with selective monitoring of masses 327–330 (22). Measured isotopomer distributions were corrected for natural ^{13}C enrichment at all masses as described previously (23), using software provided by Dr. Henri Brunengraber (Western Reserve University, Cleveland, OH). Coefficients of variation for enrichment measurements for both glucose and lactate were less than 5%, as assessed from quality control samples prepared and analyzed with the samples.

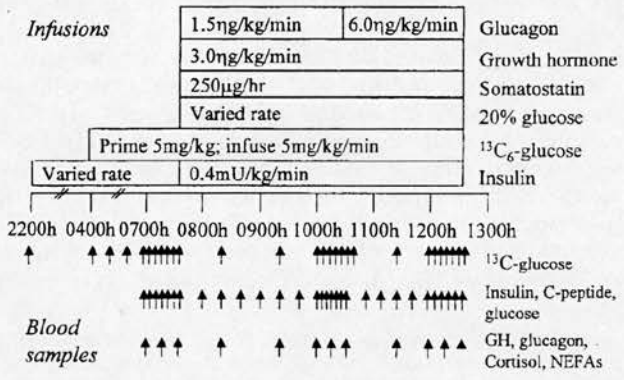

FIG. 1. Protocol for clamp study. *Arrows* indicate the timing of blood sampling.

Calculation of glucose kinetic parameters

Rates of glucose appearance (R_a) and peripheral glucose disposal (R_d) were calculated from steady state enrichment of the plasma glucose pool with [$^{13}C_6$]glucose, using mean data obtained in the basal state (0700–0730 h), during hyperinsulinemia (1000–1030 h), and with the addition of hyperglucagonemia (1200–1230 h). All enrichments during these periods were confirmed as steady state by regression coefficients for seven measurements against time not significantly different from zero. Glucose and lactate enrichments achieved in plasma at plateau were similar to those reported by Tayek and Katz (24). The glucose production rate was calculated by subtracting the glucose infusion rate from R_a. Gluconeogenesis rates were calculated at the same intervals according to the steady state formulas described by Tayek and Katz (24). Glycogenolysis was calculated as (glucose production rate) – (gluconeogenesis rate).

Statistics

Data are expressed as the mean ± SEM. The effects of carbenoxolone within groups were examined by paired *t* tests. Differences between patient groups were tested by unpaired *t* tests. Multiple regression was used to explore whether interindividual differences in the effects of carbenoxolone were attributable to differences in achieved plasma level of carbenoxolone or differences between DM patients and controls (analyzed as 0 or 1).

Results

Baseline characteristics

DM and control men were well matched for age (59 ± 3 *vs.* 58 ± 3 yr, respectively; $P = 0.94$), body mass index (29.2 ± 1.3 *vs.* 29.1 ± 0.9; $P = 0.94$), and waist/hip circumference ratio (0.95 ± 0.01 *vs.* 0.92 ± 0.03; $P = 0.43$). Glycemic control was excellent in all DM patients, so that HbA_{1c} was only marginally higher than in controls (6.8 ± 0.4% *vs.* 6.0 ± 0.1%; $P = 0.06$). HDL cholesterol was lower in DM patients (Table 1).

Effects of carbenoxolone on blood pressure, plasma electrolytes, and lipids

Tablet count and plasma carbenoxolone levels confirmed good compliance with study medication (Table 1). Carbenoxolone levels tended to be higher in DM than controls ($P = 0.09$). Carbenoxolone had the expected effects to raise blood pressure and lower plasma potassium in both groups, although the effect on plasma potassium was only statistically significant in the DM patients. In contrast, in the control group carbenoxolone decreased fasting plasma cholesterol and tended to increase HDL cholesterol; these effects were not observed in DM patients.

Effects of carbenoxolone on glucose kinetic parameters

The technical success of the clamps is shown in Fig. 2. Plasma glucose was maintained similarly close to 5.0 mM throughout in both groups with and without carbenoxolone (Fig. 2b). To achieve this insulin was infused at low doses overnight in five of the DM patients and three of the control subjects (controls after placebo, 0.3 ± 0.1 U/h; controls after carbenoxolone, 0.4 ± 0.2 U/h; DM after placebo, 1.0 ± 0.6 U/h; DM after carbenoxolone, 1.3 ± 0.3 U/h). The resulting plasma insulin levels at 0700–0730 h tended to be higher in DM patients regardless of carbenoxolone therapy (Fig. 2c). Thereafter, the anticipated degree of hyperinsulinemia was achieved by infusion of 0.4 mU/kg·min insulin, but resulting insulin concentrations were higher in DM subjects after placebo than in other groups. C Peptide levels were similar at baseline and were suppressed in all subjects during hyperinsulinemia (Fig. 2d). GH levels were similar at baseline and were clamped successfully in all participants except one DM patient after carbenoxolone whose GH level rose to more than 15 mU/liter from 1200–1230 h (Fig. 2g). Plasma glucagon levels were not different at baseline and were clamped, as intended, to physiological levels by infusion of 1.5 ng/kg·min and to high levels by infusion of 6.0 ng/kg·min (Fig. 2f). Plasma cortisol followed the normal diurnal rhythm in all groups (Fig. 2h).

Glucose was infused at variable rates during hyperinsulinemia. The rate of glucose infusion plateaued to similar rates in all groups within 1 h (Fig. 2a). There was a nonsignificant trend for higher absolute infusion rates in healthy controls after carbenoxolone consistent with increased whole body insulin sensitivity as previously reported (16). This was not attributable to differences in peripheral insulin sensitivity. Free fatty acid levels were not different at baseline and were suppressed similarly during hyperinsulinemia (Fig. 2e). R_d (Fig. 3a) was stimulated, as expected, by insulin and rose further with the addition of hyperglucagonemia, especially in control subjects, but was unaffected by carbenoxolone in either group.

Glucose production rates were not different at baseline, were suppressed during hyperinsulinemia, and were stimulated during hyperglucagonemia (Fig. 3b). These changes in glucose production during the clamp were associated with the expected changes in gluconeogenesis (Fig. 3c) and gly-

TABLE 1. Effect of carbenoloxone on clinical characteristics and biochemistry

	Controls		Diabetic patients	
	Placebo	Carbenoxolone	Placebo	Carbenoxolone
Weight (kg)	89.5 ± 5.9	89.9 ± 5.7	83.0 ± 5.8	84.2 ± 5.6
Systolic blood pressure (mm Hg)	132 ± 8	149 ± 6[b]	130 ± 6	148 ± 6[b]
Diastolic blood pressure (mm Hg)	79 ± 4	84 ± 4[a]	78 ± 5	84 ± 6[a]
Plasma potassium (mM)	4.38 ± 0.08	4.12 ± 0.14	4.30 ± 0.10	4.07 ± 0.12[a]
Plasma total cholesterol (mM)	5.25 ± 0.34	4.78 ± 0.40[b]	4.28 ± 0.70	4.33 ± 0.33
HDL cholesterol (mM)	1.28 ± 0.15	1.42 ± 0.15	0.89 ± 0.07[c]	0.95 ± 0.09[c]
Plasma triglycerides (mM)	1.75 ± 0.62	1.16 ± 0.37	1.27 ± 0.37	1.00 ± 0.21
Plasma carbenoloxone (μg/liter)	All <10	46.8 ± 11.8[b]	All <10	88.3 ± 18.5[b]

Data are mean ± SE.
[a] $P < 0.05$ and [b] $P < 0.01$ in paired Student's *t* test *vs.* placebo.
[c] $P < 0.05$ in Student's *t* test *vs.* control group.

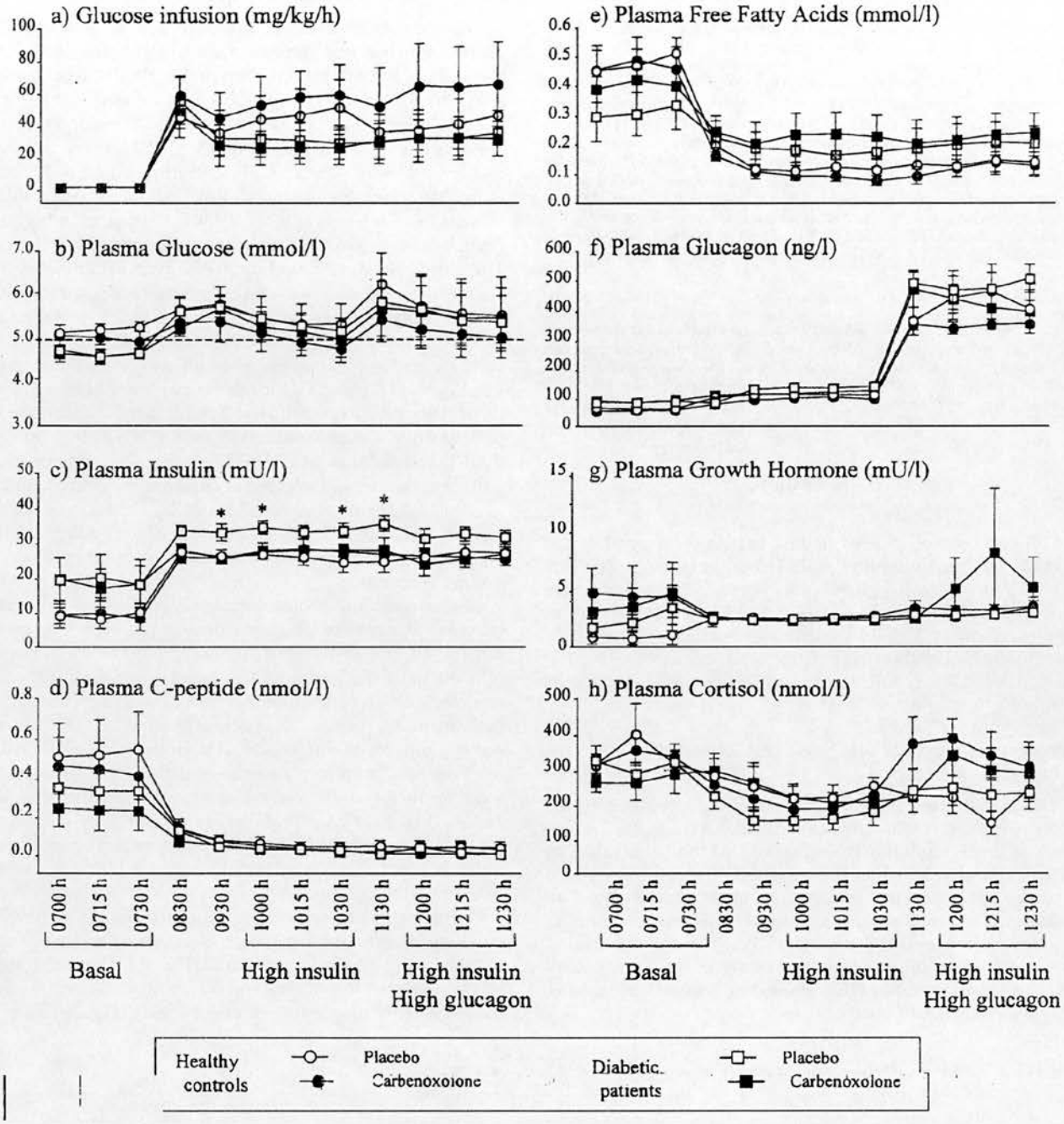

FIG. 2. Direct measurements during clamp study. Data are the mean ± SEM. For clarity, only limited time points are shown for each measurement. ○ and ●, Healthy controls; □ and ■, diabetic patients. ○ and □, After placebo; ● and ■, after carbenoxolone. *, $P < 0.05$ *vs.* all other groups (by unpaired *t* tests *vs.* controls and by paired *t* tests *vs.* DM group during carbenoxolone treatment).

cogenolysis (Fig. 3d), both of which were suppressed by hyperinsulinemia and stimulated by hyperglucagonemia. By contrast with the lack of effect on peripheral glucose uptake, carbenoxolone prevented the increase in the glucose production rate during hyperglucagonemia in DM subjects only. This was attributable to reduced glycogenolysis, with no significant difference in gluconeogenesis.

The influence of interindividual variations in plasma carbenoxolone concentrations was investigated for each of the variables that were significantly different between carbenoxolone and placebo phases in either group. In Pearson simple correlations, plasma levels of carbenoxolone were not significantly associated with the difference between measurements during carbenoxolone and placebo phases. Mul-

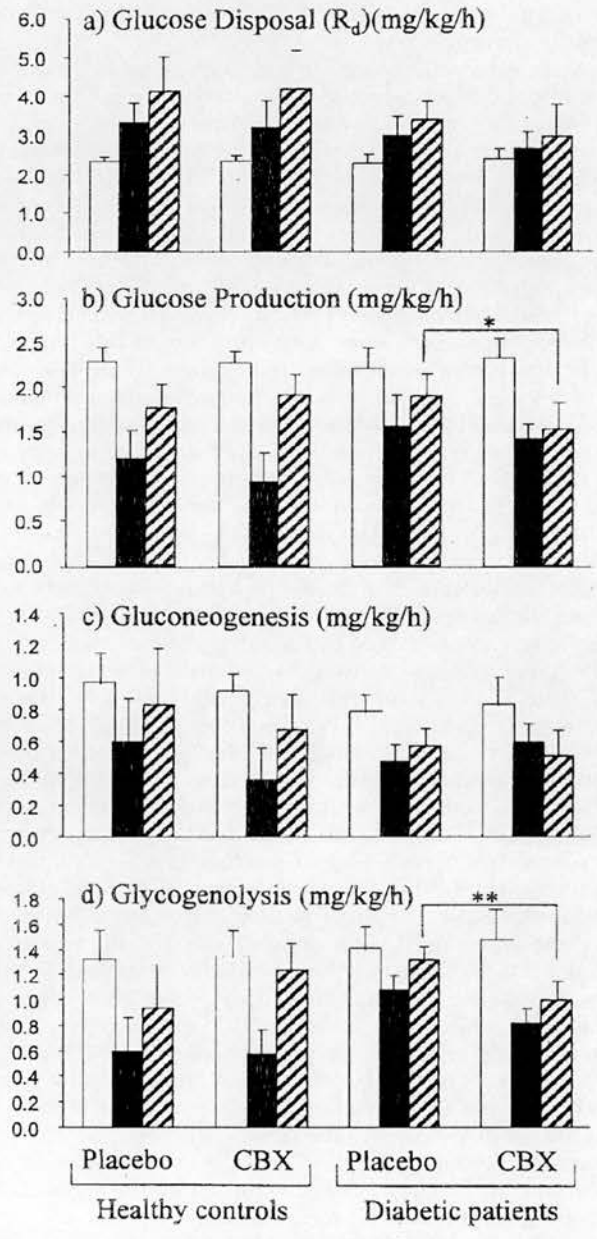

FIG. 3. Kinetic parameters derived from [$^{13}C_6$]glucose tracer measurements. Data are the mean ± SEM. □, Basal measurements from 0700–0730 h; ■, measurements during hyperinsulinemia from 1000–1030 h; ▨, measurements during hyperinsulinemia and hyperglucagonemia from 1200–1230 h. *, $P < 0.05$; **, $P < 0.01$ (between the groups indicated, by paired t tests).

tiple regression was employed to explore whether differences in the effects of carbenoxolone between DM and controls could be accounted for by differences in plasma carbenoxolone levels. Explanatory variables were plasma carbenoxolone concentration and diagnosis (DM or control, coded as 0 and 1). These models did not show any independent effect of plasma carbenoxolone concentration.

Discussion

Previous research in mice, rats, and healthy humans suggests that inhibition of 11β-HSD1 lowers intrahepatic glucocorticoid concentrations and thereby reduces hepatic glucose production and enhances lipid catabolism (1). In addition, more recent evidence suggests that inhibition of 11β-HSD1 in adipose tissue will increase peripheral glucose uptake and suppress lipolysis (5). This report extends previous studies using the nonselective 11β-HSD inhibitor, carbenoxolone (10, 14). We examined in detail its site of action on glucose metabolism in healthy men and tested its effects in patients with type 2 diabetes, a patient group that might be expected to benefit from any future development of selective 11β-HSD1 inhibitors. We employed a detailed protocol to control for variables that are sometimes neglected during euglycemic clamp studies, including overnight preparation of subjects to avoid effects of baseline hyperglycemia (25, 26), clamping of GH and glucagon levels, and stable isotope tracer measurement of gluconeogenesis. We showed that 1 wk of carbenoxolone administration decreased glucagon-stimulated glucose production and glycogenolysis in diabetic, but not healthy, subjects and decreased total cholesterol in healthy, but not diabetic, subjects. Carbenoxolone had no effect on gluconeogenesis, peripheral glucose uptake, or insulin-mediated suppression of plasma free fatty acids. These observations reinforce the potential value of 11β-HSD1 inhibitors in enhancing hepatic insulin sensitivity and lipid catabolism.

An important consideration in designing this study to test the utility of 11β-HSD1 inhibition in metabolic disease was raised by observations that there are tissue-specific alterations in enzyme activity in obesity. Thus, 11β-HSD1 is increased in adipose tissue, but decreased in liver in obesity (6–8, 17, 27). In contrast, lean patients with type 2 diabetes have normal adipose 11β-HSD1 and less marked downregulation of hepatic conversion of cortisone to cortisol (18, 28). To avoid the potential confounding effects of obesity and to exclude any unknown effects of oral hypoglycemic or antihypertensive agents, we selected nonobese normotensive patients with type 2 diabetes controlled by dietary therapy alone. The result was that patients in this study were not typical of type 2 diabetes. Indeed, they had near-normal blood glucose and HbA_{1c} levels, a small requirement for overnight insulin infusion to obtain fasting euglycemia, and only minor differences in plasma lipids. After overnight euglycemia with insulin infusion as required, glucose production, free fatty acids, and glucagon levels were not elevated in these diabetic patients, and glucose disposal was not measurably impaired. Nonetheless, the effects of carbenoxolone differed between healthy controls and diabetic patients; an effect on cholesterol was only evident in healthy controls, and measurable effects on glucose production were only evident in diabetic patients.

In a previous study we showed that the same regime of carbenoxolone administration to healthy men resulted in enhanced insulin sensitivity, as measured by increased glucose infusion rate during a hyperinsulinemic clamp (16). A key difference, however, is that the previous study was performed with a higher insulin infusion rate and achieved

higher concentrations (~70 mU/liter compared with ~30 mU/liter here) designed to examine effects on insulin-stimulated glucose uptake rather than glucose production (29). Also, GH and glucagon levels were not clamped previously, and the participants were younger. In the current study there was a trend for a similar magnitude of increase in glucose infusion rate in healthy controls (means differed by ~7% previously and by ~17% here), but it did not reach statistical significance. Glucose production was marginally, but not significantly, lower in healthy men after carbenoxolone treatment in the current study. This contrasts with the statistically significant effects of carbenoxolone on glucose kinetics in diabetic patients. By analogy with other insulin-sensitizing therapies it might be anticipated that the effects of carbenoxolone would be smaller in healthy controls than in diabetic patients, because, for example, troglitazone induced around twice the increase in insulin sensitivity in diabetic patients (30) as it did in healthy men (31). Further, plasma carbenoxolone levels tended to be higher in the diabetic patients than in controls, so that the effect of carbenoxolone could have been underestimated in control subjects. Single measurements of plasma carbenoxolone concentrations were included in this study principally as a qualitative assessment of compliance, and more detailed pharmacokinetic studies would be required to confirm that this difference did not occur by chance. Importantly, however, in multiple regression analysis the variations in carbenoxolone concentrations between individuals did not account for different effects of carbenoxolone in DM patients and controls. Finally, insulin levels during the clamp studies were higher in the diabetic patients during placebo therapy than in all other groups, which may lead to underestimation of the effects of carbenoxolone in the diabetic patients. Against this background, it is unclear whether quantitative or qualitative differences explain the discrepancies between the effects of carbenoxolone in health and diabetes, although we suspect the former.

This is the first report of the effects of carbenoxolone, or any 11β-HSD inhibitor, in diabetic patients. It shows that carbenoxolone affects glucose production, as inferred indirectly from our previous report (16), but the mechanism of the effect was not expected. In 11β-HSD1 knockout mice a key feature is impaired up-regulation of gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase, on fasting (2). Glucocorticoids are known to oppose the effect of insulin in regulating the expression of gluconeogenic enzymes (32). However, carbenoxolone did not alter gluconeogenesis after overnight fast, during hyperinsulinemia, or during hyperglucagonemia. One consideration in this paradox is that the contribution of the kidney to gluconeogenesis in man remains unquantified. By inhibiting inactivation of cortisol by 11β-HSD2 in kidney (14) carbenoxolone increases intrarenal cortisol concentrations, which might enhance renal gluconeogenesis in compensation. To resolve this will require studies either with selective 11β-HSD1 inhibitors or with cannulation of hepatic and/or renal vein. The kidney is not, however, a major site of glycogen storage. Glucocorticoids have complex effects on glycogenic and glycogenolytic enzymes, which predict increased turnover and amplification of the effect of other signals (32, 33). Thus, the observation that carbenoxolone attenuated net glucagon-induced glycogenolysis is consistent with lowering of intrahepatic cortisol concentrations.

A more recently recognized consequence of changes in intrahepatic glucocorticoid concentrations is the effect on lipid metabolism (3). The effects of carbenoxolone in the liver are the most likely explanation for the decrease in total cholesterol observed in healthy controls. In 11β-HSD1 knockout mice hepatic lipid catabolism is markedly increased, while synthesis is apparently normal, resulting in reduced serum triglycerides and total cholesterol (3). In addition, altered apolipoprotein A1 expression in these animals may account for higher HDL cholesterol (3). However, the importance of enhanced lipid catabolism in man and comparison of effects in healthy controls and diabetic patients should be reassessed with a longer duration of carbenoxolone administration, because plasma lipids take several weeks to reequilibrate after the introduction of conventional lipid-lowering therapy.

Liquorice derivatives, such as carbenoxolone and glycyrrhetinic acid, are potent inhibitors of both isozymes of 11β-HSD *in vitro* and in cell culture (10, 34–36). However, *in vivo* they have inconsistent effects, probably because of pharmacokinetic differences in access to tissues. Thus, carbenoxolone, but not glycyrrhetinic acid, inhibits hepatic 11β-HSD1 *in vivo* in man, as judged by impaired generation of cortisol after an oral dose of cortisone. In animals, *in vivo* inhibition of 11β-HSDs with carbenoxolone in other tissues is also inconsistent, for example varying between different regions of the central nervous system (37, 38). Indeed, in Zucker obese rats *in vivo* administration of carbenoxolone inhibits 11β-HSD1 in liver, but not in adipose tissue (Livingstone, D. E. W., *et al.*, unpublished observations). In the current study relatively modest hyperinsulinemia was employed to approximate the ED_{50} for suppression of hepatic glucose production (29). For these reasons the positive effects of carbenoxolone on hepatic carbohydrate and lipid metabolism but lack of effect on peripheral glucose uptake in both the current and previous study (16) do not allow the conclusion that inhibition of 11β-HSD1 in extrahepatic tissues, notably adipose tissue, would not be beneficial. However, the acute effects of carbenoxolone on hepatic insulin sensitivity were of modest magnitude in the lean group of patients studied here. For more substantial effects on glucose tolerance and glycemic control in patients with diabetes, it appears likely that 11β-HSD1 inhibitors will be required to inhibit glucocorticoid regeneration in adipose tissue as well as liver. Reducing cortisol action in adipose tissue may then provide an increase in peripheral glucose disposal in addition to the reduced glucose production observed with carbenoxolone. Further, given the up-regulation of adipose 11β-HSD1 in obesity (6–8), but not in lean patients with type 2 diabetes (18), inhibition of adipose is likely to be of the most benefit in obese patients.

In summary, these studies with a nonselective 11β-HSD inhibitor illustrate the potential value of inhibition of 11β-HSD1 in lean hyperglycemic patients. It will now be important to establish whether similar benefits can be obtained in obese patients and patients with dyslipidemia. However, by inhibiting renal 11β-HSD2, carbenoxolone has unacceptable long-term side-effects, including raising blood pressure, so that exploiting this approach for useful therapy will require

either simultaneous blockade of renal mineralocorticoid receptors or the long-awaited development of selective 11β-HSD1 inhibitors.

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Address all correspondence and requests for reprints to: Prof. Brian R. Walker, University of Edinburgh, Endocrinology Unit, Department of Medical Sciences, Western General Hospital, Edinburgh, United Kingdom EH4 2XU. E-mail: b.walker@ed.ac.uk.

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Present address for R.C.A.: University of Bristol, Research Center for Neuroendocrinology, Bristol Royal Infirmary, Bristol, United Kingdom BS2 8HW.

Present address for O.R.: Department of Anesthesiology, KARO, Karolinska Institute, Huddinge Hospital, 14186 Hudinge, Sweden.

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Abnormal Cortisol Metabolism and Tissue Sensitivity to Cortisol in Patients with Glucose Intolerance

ROBERT C. ANDREWS, OLIVE HERLIHY, DAWN E. W. LIVINGSTONE, RUTH ANDREW, AND BRIAN R. WALKER

University of Edinburgh, Endocrinology Unit, Department of Medical Sciences, Western General Hospital, Edinburgh EH4 2XU, United Kingdom

Recent evidence suggests that increased cortisol secretion, altered cortisol metabolism, and/or increased tissue sensitivity to cortisol may link insulin resistance, hypertension, and obesity. Whether these changes are important in type 2 diabetes mellitus (DM) is unknown.

We performed an integrated assessment of glucocorticoid secretion, metabolism, and action in 25 unmedicated lean male patients with hyperglycemia (20 with type 2 diabetes and 5 with impaired glucose intolerance by World Health Organization criteria) and 25 healthy men, carefully matched for body mass index, age, and blood pressure. Data are mean ± SE. Patients with hyperglycemia (DM) had higher HbA_{1c} (6.9 ± 0.2% *vs.* 6.0 ± 0.1%, $P < 0.0001$) and triglycerides. Cortisol secretion was not different, as judged by 0900 h plasma cortisol and 24 h total urinary cortisol metabolites. However, the proportion of cortisol excreted as 5α- and 5β-reduced metabolites was increased in DM patients. Following an oral dose of cortisone 25 mg, generation of plasma cortisol by hepatic 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD 1) was impaired in DM patients (area under the curve, 3617 ± 281 nM.2 h *vs.* 4475 ± 228; $P < 0.005$). In contrast, in sc gluteal fat biopsies from 17 subjects (5 DM and 12 controls) *in vitro* 11β-HSD 1 activity was not different (area under the curve, 128 ± 56% conversion.30 h DM *vs.* 119 ± 21, $P = 0.86$). Sensitivity to glucocorticoids was increased in DM patients both centrally (0900 h plasma cortisol after overnight 250 μg oral dexamethasone 172 ± 16 nM *vs.* 238 ± 20 nM, $P < 0.01$) and peripherally (more intense forearm dermal blanching following overnight topical beclomethasone; 0.56 ± 0.92 ratio to vehicle *vs.* 0.82 ± 0.69, $P < 0.05$).

In summary, in patients with glucose intolerance, cortisol secretion, although normal, is inappropriately high given enhanced central and peripheral sensitivity to glucocorticoids. Normal 11β-HSD 1 activity in adipose tissue with impaired hepatic conversion of cortisone to cortisol suggests that tissue-specific changes in 11β-HSD 1 activity in hyperglycemia differ from those in primary obesity but may still be susceptible to pharmacological inhibition of the enzyme to reduce intracellular cortisol concentrations. Thus, altered cortisol action occurs not only in obesity and hypertension but also in glucose intolerance, and could therefore contribute to the link between these multiple cardiovascular risk factors. (*J Clin Endocrinol Metab* 87: 5587–5593, 2002)

HYPERTENSION, OBESITY, coronary heart disease, and hyperlipidemia are extremely common in patients with glucose intolerance or type 2 diabetes mellitus (DM). These are associated with insulin resistance (1) in what is referred to as the Metabolic Syndrome, but the reasons for the associations between features of this syndrome remain obscure. A similar association of cardiovascular risk factors and insulin resistance occurs in Cushing's syndrome, due to elevated circulating glucocorticoids. It has been proposed that subtle abnormalities in cortisol action are a missing link between these factors in patients with the Metabolic Syndrome (2–4).

The hypothalamic-pituitary-adrenal (HPA) axis controls the secretion of cortisol, with excessive secretion being inhibited by negative feedback. In addition, tissue cortisol concentrations are controlled by the relative activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD 1), which converts inactive cortisone to active cortisol, and 11β-HSD 2, which converts cortisol to cortisone (5). Tissues may thus be exposed to a relative excess of cortisol without any increase in cortisol secretion or plasma cortisol concentrations. The potential importance of 11β-HSD 1 in the Metabolic Syndrome has been illustrated by recent experiments in mice. 11β-HSD 1 knockout mice are protected from insulin resistance, hyperglycemia, and dyslipidemia (6, 7), whereas mice overexpressing 11β-HSD 1 selectively in adipose tissue under the AP2 promoter are centrally obese, hyperglycemic, and hyperlipidemic (8). Similarly, variations in glucocorticoid receptor expression can influence tissue responses independently of circulating glucocorticoid concentrations (9).

A number of cross-sectional cohort studies have found that higher 0900 h plasma cortisol and/or increased total 24 h urinary cortisol metabolite excretion is associated with insulin resistance, high blood pressure, hyperlipidemia, and hyperglycemia (4, 10–16). Negative feedback control of the HPA axis appears normal (17), however, and the activation of the HPA axis in men with the Metabolic Syndrome may reflect an increase in central drive to the hypothalamus (18, 19). Obese individuals also show subtle changes in cortisol activity with an increase in 24 h cortisol secretion despite normal (20) or enhanced (21) feedback sensitivity (as shown by suppression of plasma cortisol to 250 μg of dexamethasone). However, plasma cortisol concentrations are not elevated, perhaps because peripheral metabolic clearance of cortisol, for example by 5α-reductase (22), is increased (23).

Tissue responses to glucocorticoids are also altered in patients with features of the Metabolic Syndrome. In case-

Abbreviations: DM, Type 2 diabetes mellitus or impaired glucose tolerance; HPA, hypothalamic-pituitary-adrenal; 11β-HSD, 11β-hydroxysteroid dehydrogenase; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

control studies, individuals with essential hypertension have reduced inactivation of cortisol by 11β-HSD 2 (24–26) and enhanced glucocorticoid-receptor-dependent tissue sensitivity to cortisol (as judged by enhanced dermal vasoconstriction following topical glucocorticoid application) (13, 17). In obesity, 11β-HSD 2 activity and dermal glucocorticoid sensitivity are not enhanced, but there are tissue-specific changes in 11β-HSD 1 activity, resulting in less reactivation of cortisone to cortisol in liver, but enhanced reactivation in sc abdominal adipose (20, 27, 28). Importantly, the magnitude of increase in 11β-HSD 1 activity in adipose tissue of obese men is similar (about 3-fold) as results in dramatic obesity and hyperglycemia in transgenic mice with 11β-HSD 1 overexpression in adipose tissue (8). This observation has reinforced the concept that inhibition of 11β-HSD 1 would be of therapeutic benefit in patients with the Metabolic Syndrome (29).

Against this background, it is important to know whether abnormalities in cortisol secretion, metabolism, or sensitivity exist in patients with type 2 diabetes or glucose intolerance, but this may be difficult to establish because these differences are confounded by contrasting effects of coexisting hypertension and obesity (30). Previous studies of cortisol in patients with DM have been conducted in heterogeneous groups with type 1 and type 2 diabetes (31–38), or have been inadequately controlled for confounding factors of sex, coexisting obesity, hypertension, poor glycemic control, and diabetic complications, making interpretation difficult (39–44). Moreover, no previous studies have examined tissue responses to glucocorticoids in patients with diabetes, or attempted to dissect tissue-specific changes in cortisol metabolism. This study set strict criteria for patient selection to examine cortisol secretion, metabolism and sensitivity in nonobese, normotensive, diet-controlled male patients with DM or impaired glucose tolerance.

Subjects and Methods

Participants

We studied 25 men with DM or impaired glucose tolerance (as defined by WHO criteria for oral glucose tolerance tests) recruited from our clinic, and 25 normal healthy controls recruited by advertisement. All patients were controlled by diet alone, without oral hypoglycemic agents or insulin, and were free of clinical or biochemical evidence of retinopathy, nephropathy, and neuropathy at last annual review. Exclusion criteria included therapy for any other medical conditions, major psychiatric disorder, weight loss more than 5 kg in the previous 3 months, blood pressure more than 160/90 mm Hg, body mass index more than 32 kg/m^2, glucocorticoid therapy by any route in previous 3 months, or abnormal renal or thyroid function on biochemical screening. Control subjects were matched for weight, height, body mass index, and blood pressure. Local ethical committee approval and written informed consent were obtained.

Protocol

Participants attended on one afternoon without fasting for a medical examination, measurement of sitting blood pressure (using a Takeda UA-751 sphygmomanometer), height, and weight. Blood was obtained for full blood count, urea and electrolytes, HbA_{1c}, liver function tests, thyroid function tests, cholesterol, and triglycerides. Beclomethasone dipropionate was then applied to the forearm and subjects returned the following morning for assessment of dermal blanching (see below). Following this visit, subjects collected a 24-h urine sample for total cortisol metabolites.

On a second occasion, participants attended at 0830 h having fasted from 2300 h the previous evening. They lay supine; an iv cannula was sited and blood was taken 30 min later for cortisol, cortisol binding globulin, glucose, and insulin.

On a third occasion, participants took 250 μg dexamethasone (Decadron, Merck, West Drayton, UK) by mouth at 2300 h and fasted until attending the following morning at 0830 h. An iv cannula was sited, and 30 min later blood was taken for cortisol estimation. Participants then took 25 mg cortisone acetate (Cortisyl, Hoechst Marion Roussel, Inc., Uxbridge, UK) by mouth and blood was taken for cortisol every 15 min for 2 h.

In vitro adipose 11β-HSD 1 activity

Seventeen subjects (5 DM and 12 controls) consented to return for a 500 mg sc fat biopsy to be taken from the gluteal region under local anesthesia. Subcutaneous fat was frozen immediately at −70 C. After thawing, it was homogenized in Krebs buffer at pH 7.4 and 750 μg/ml protein was incubated at 37 C with nicotinamide adenine dinucleotide phosphate (2 mM) and 1,2,6,7-$^3H_4$-cortisol (100 nM) for 30 h. Samples were taken at 3, 6, 20, and 30 h for separation of cortisol and cortisone by HPLC with on-line liquid scintillation detection (20). 11β-HSD 1 activity was measured in the dehydrogenase direction (*i.e.* cortisol to cortisone, rather than reductase cortisone to cortisol) because dehydrogenase activity is more stable than reductase activity *in vitro*, and because dehydrogenase is the preferred reaction when the enzyme is liberated from its intracellular environment (5). Under these conditions, the conversion of cortisol to cortisone is proportionate to the total protein added, and therefore reflects 11β-HSD 1 protein concentrations in the biopsy sample.

Dermal vasoconstrictor response to glucocorticoids

This was performed as previously described (45). In brief, 50 μl beclomethasone dipropionate (Sigma, St. Louis, MO) at 0, 1, 5, 10, 100, or 1000 μg/ml in 95% ethanol were applied in random order into 6 circles of 20-mm diameter on the volar surface of the nondominant forearm. After the ethanol had evaporated, all sites were occluded with Saran wrap (Dow Corning, Midland, MI) and left for 16–18 h. At 0800 h the next day, the bandage was removed. An hour later, the intensity of the blanching was read by an observer who was blind to the order of application using a reflectance spectrophotometer (Erythemameter, Diastron Ltd., Andover, UK). This device measures the ratio of red/green light reflected from the skin surface, called the erythema index. Because red reflects oxyhemoglobin concentration and green reflects melanin concentrations, the erythema index corrects for variations in skin color between individuals. The erythema index for each test site was divided by the erythema index for the site treated with vehicle alone to produce a blanching index. The blanching index corrects for the nonspecific variations in skin color that occur in different environments in the same individuals. A lower blanching index indicates more intense blanching.

Laboratory analyses

Plasma and urine samples were stored at −80 C and −20 C, respectively.

RIAs were used to measure plasma cortisol (46), dexamethasone (Cozart Bioscience, Abingdon, UK), and corticosteroid binding globulin (Medgenix Diagnostics, Fleurus, Belgium). Insulin was measured by enzyme immunoassay (Eurogenetics UK Ltd., Hampton, UK). Glucose was measured by an enzymatic technique (Cabas Mira Plus, Roche Molecular Biochemicals, Mannheim, Germany). Ion exchange HPLC was used to measure the HbA_{1c} (Variant 11, Bio-Rad Laboratories, Inc., Hercules, CA).

Cortisol and its metabolites were measured in urine by electron impact gas chromatography/mass spectrometry following Sep-pak C18 extraction, hydrolysis with β-glucuronidase, and formation of the methoximetrimethylsilyl derivatives (47). Epi-cortisol and epi-tetrahydrocortisol were used as internal standards. Total cortisol metabolite excretion was calculated as tetrahydrocortisols (THFs) + tetrahydrocortisone (THE) + cortols + cortolones (48). Relative metabolism by 5α and 5β-reductases were inferred from the 5β-THF/5α-THF ratio. A-ring reduction of cortisol was inferred from the ratios of THFs/cortisol (49) and 5β-reductase activity

from the ratio of THE/cortisone. Whole-body equilibrium between cortisol and cortisone, determined by the balance of tissue-specific activities of 11β-reductase and 11β-dehydrogenase activities, was inferred from the ratio of THFs/THE. Renal 11β-dehydrogenase activity was inferred from the urinary free cortisol/cortisone ratio (47, 50).

Statistics

Data are expressed as means ± SE. All groups were compared by Student's *t* test apart from the urine data, which were compared by Mann Whitney *U* test, as these data were not normally distributed. Profiles of cortisol and dermal vasoconstriction were compared by repeated measures ANOVA.

Results

Baseline characteristics

Characteristics of participants are shown in Table 1. The groups were well matched for anthropometric, clinical, and biochemical variables except that diabetic patients had higher fasting plasma glucose, HbA_{1c}, and triglycerides than controls. Corticosteroid binding globulin and albumin did not differ between the groups, so only total plasma cortisol was used in further analysis.

HPA axis activity

Fasting morning plasma cortisol (Table 1) and total urinary cortisol metabolite excretion rate (Table 2) were not different between groups. However, the morning after 250 μg oral dexamethasone, plasma cortisol was lower in diabetic patients (Table 1). This could not be attributed to differences in dexamethasone concentrations (Table 1).

Cortisol metabolism

Although total cortisol metabolite excretion was not different between groups, there were changes in relative metabolite excretion (Table 2). DM patients excreted less unmetabolized cortisol ($P < 0.03$) and cortisone ($P = 0.07$) and tended to excrete more as 5β-THF ($P = 0.07$). As a result, ratios reflecting 5β-reduction of cortisol (5β-THF/cortisol, $P < 0.001$) and cortisone (THE/cortisone, $P < 0.005$) were increased in DM patients, and there was a trend for increased 5α-reduction of cortisol (5α-THF/cortisol). Absolute excretion of other metabolites, and ratios reflecting 11β-HSD2 (cortisol/cortisone) and overall 11β-HSDs (THFs/THE), were not different.

Hepatic 11β-HSD 1 activity, measured as conversion of orally administered cortisone to cortisol, was impaired in the DM group (Fig. 1; area under curve, 3617 ± 281 nM *vs.* 4475 ± 228 nM; ANOVA $P < 0.005$), with an increase in time taken to reach maximum plasma cortisol (111 ± 3 min *vs.* 100 ± 4 min, $P < 0.05$). By simple regression, there was no relationship between hepatic 11β-HSD 1 and any individual urinary cortisol metabolite or ratio. Adipose *in vitro* 11β-HSD 1 activity was no different between the two groups (Fig. 2; area under the curve, 119 ± 21% controls *vs.* 128 ± 56% DM, $P = 0.8$).

Peripheral tissue sensitivity to glucocorticoids

Dermal vasoconstriction to topical beclomethasone dipropionate was more intense in the DM group than in the control group (Fig. 3).

Discussion

This study demonstrates that nonobese normotensive men with hyperglycemia exhibit abnormalities in cortisol activity. The differences in cortisol metabolism and tissue sensitivity were more striking than any differences in HPA axis function. Specifically, these patients with DM or impaired glucose tolerance show: 1) normal cortisol secretion and circulating levels in the face of enhanced negative feedback sensitivity (as measured with dexamethasone); 2) enhanced *in vivo* peripheral tissue sensitivity to glucocorticoids (as measured by dermal blanching); 3) impaired hepatic 11β-HSD 1 activity but normal adipose 11β-HSD 1 activity, suggesting tissue-specific alterations in 11β-HSD 1 activity; and 4) increased relative excretion of A-ring reduced metabolites of cortisol. These findings suggest that isolated hyperglycemia is associated with some, but not all, of the changes in cortisol metabolism and action that have been observed in subjects with hypertension or obesity and the Metabolic Syndrome. This

TABLE 1. Clinical characteristics and biochemistry

	Controls (n = 25)	Hyperglycemic patients (n = 25)	Student's *t* test *P*
Age (yr)	59 ± 2	58 ± 2	0.59
Body mass index (kg/m^2)	27.2 ± 0.5	27.6 ± 0.6	0.56
Systolic blood pressure (mm Hg)	130 ± 3	131 ± 2	0.66
Diastolic blood pressure (mm Hg)	78 ± 2	78 ± 1	0.78
Plasma creatinine (μM)	89.2 ± 2.6	89.8 ± 2.7	0.87
HbA_{1c} (%)	6.0 ± 0.1	6.9 ± 0.2	<0.0001
Fasting plasma glucose (mM)	5.7 ± 0.2	8.2 ± 0.6	<0.0002
Fasting plasma insulin (mU/liter)	19.6 ± 4.2	21.4 ± 3.0	0.74
Plasma triglycerides (mM)	2.1 ± 0.2	3.5 ± 0.6	<0.05
Total plasma cholesterol (mM)	5.6 ± 0.2	5.6 ± 0.2	0.92
Plasma albumin (g/liter)	42 ± 0.5	42 ± 0.8	0.50
0900 h plasma cortisol (nM)	420 ± 30	428 ± 24	0.85
0900 h plasma cortisol postdexamethasone 250 μg (nM)	238 ± 20	172 ± 16	<0.01
Plasma dexamethasone postdexamethasone 250 μg (ng/ml)	0.40 ± 0.11	0.41 ± 0.05	0.92
Plasma cortisol binding globulin (μg/ml)	31.9 ± 1.4	28.8 ± 1.7	0.16

Data are mean ± SE.

TABLE 2. Urinary cortisol metabolites

	Controls (n = 25)	Hyperglycemic patients (n = 25)	Mann-Whitney *U* test *P*
Cortisol	100 ± 6	84 ± 9	0.03
Cortisone	112 ± 14	75 ± 6	0.07
5α-tetrahydrocortisol (5α-THF)	1197 ± 153	1196 ± 104	0.66
5β-tetrahydrocortisol (5β-THF)	1036 ± 95	1264 ± 90	0.07
Tetrahydrocortisone (THE)	2218 ± 685	1433 ± 105	0.13
Total cortisol metabolites[a]	7691 ± 1336	7535 ± 1514	0.80
(5α-THF + 5β-THF)/THE	1.94 ± 0.19	1.80 ± 0.11	0.47
5β-THF/5α-THF	1.06 ± 0.11	1.19 ± 0.11	0.29
Cortisol/cortisone	1.09 ± 0.08	1.13 ± 0.05	0.97
5β-THF/cortisol	11.0 ± 1.0	16.4 ± 1.0	0.001
5α-THF/cortisol	12.6 ± 1.6	16.5 ± 1.6	0.07
THE/cortisone	15.8 ± 2.2	20.4 ± 1.2	0.005

Results for each steroid are micrograms per day; other results are ratios; mean ± SE.
[a] Total cortisol metabolites = 5α-THF + 5β-THF + THE + cortols + cortolones.

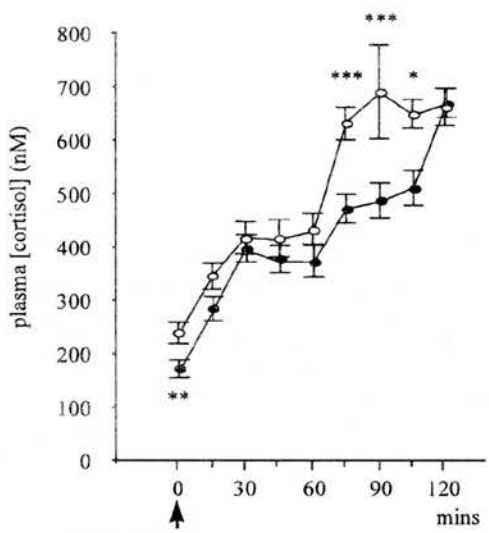

FIG. 1. *In vivo* hepatic 11β-HSD 1 activity: conversion of oral cortisone to plasma cortisol. Subjects received oral dexamethasone 250 μg at 2300 h the previous evening and 25 mg oral cortisone at 0900 h (time 0; *arrow*). Data are mean ± SE for controls (*open symbols*, n = 25) and DM patients (filled symbols, n = 25). By repeated measures two-way ANOVA, plasma cortisol was lower in diabetics ($P < 0.005$). *Asterisks* show *post hoc* comparisons at each time point by least squares difference tests: *, $P < 0.02$; **, $P < 0.01$; ***, $P < 0.0001$.

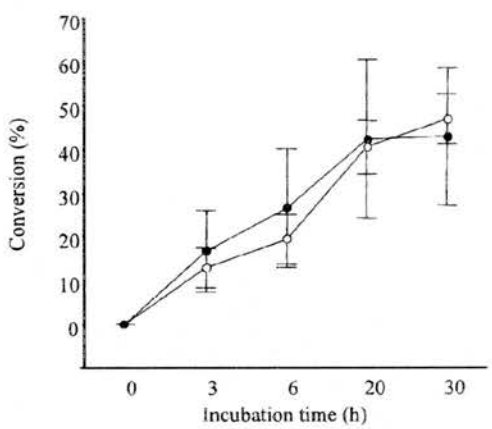

FIG. 2. *In vitro* 11β-HSD 1 activity in sc fat biopsy. Data are mean ± SE for % conversion of cortisol to cortisone at fixed protein concentrations for control subjects (*open symbol*, n = 12) and DM patients (*filled symbols*, n = 5). By repeated measures two-way ANOVA, there was no difference between the two groups ($P = 0.8$).

has implications for understanding underlying mechanisms predisposing to hyperglycemia, determinants of altered glucocorticoid signaling, and therapeutic opportunities to manipulate cortisol action to improve metabolic control in DM.

Previous studies of cortisol in patients with diabetes have focused on individuals with type 1 diabetes. These showed increased plasma and urinary free cortisol levels among patients with poor glycemic control and/or diabetic complications (40–42, 51–53), but these abnormalities were less marked in well controlled uncomplicated patients (40, 54). A number of older studies looking at patients with both type 1 and type 2 diabetes found less consistent abnormalities (34–37, 55) but again showed higher plasma cortisol concentrations in patients with complications (32, 37). Few studies have included only patients with type 2 diabetes and these did not show altered secretion (44, 57) or circulating levels (58) of cortisol. However, obesity (22), gender and blood pressure (13) affect cortisol secretion and metabolism; these factors were not controlled for in previous studies of patients with diabetes. Against this background, the strength of the current study is the careful matching of controls and patients with type 2 diabetes or impaired glucose tolerance, the focus on men only, and the exclusion of patients with obesity, hypertension, and diabetes complications. The aim was to isolate the influence of abnormal insulin action and hyperglycemia from these confounding effects. This was achieved in so far as the only detected differences in baseline characteristics between patients and controls were in fasting plasma glucose, HbA_{1C}, and triglyceride levels. Fasting insulin levels were not different between groups, consistent with relative insulin deficiency in the hyperglycemic patients. To achieve this close matching with healthy controls, however, necessitated selection of a group of patients with extremely good metabolic control of their hyperglycemia. As a result, the current study may underestimate effects of hyperglycemia *per se*, but nonetheless will detect differences that are intrinsic to patients who have pancreatic β-cell dysfunction.

Other studies have used conventional techniques to assess

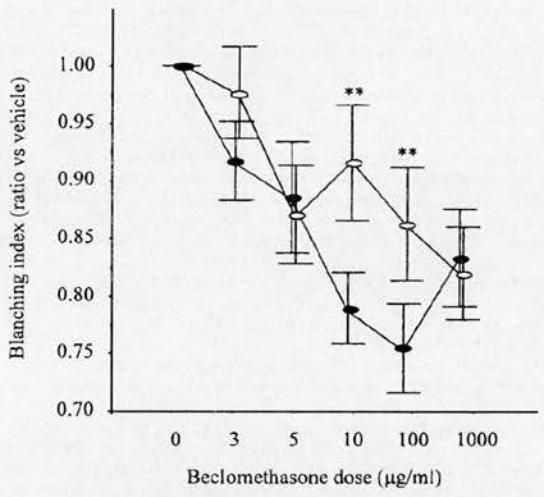

FIG. 3. Dermal vasoconstriction following topical beclomethasone dipropionate. Blanching index was recorded following overnight topical application of beclomethasone dipropionate. A lower index indicates more intense blanching. Data are mean ± SE for controls (*open symbols*, n = 25) and diabetics (*filled symbols*, n = 25). By repeated measures two-way ANOVA, blanching was greater in diabetics ($P = 0.05$). *Asterisks* show *post hoc* comparisons at each dose by least squares difference tests: **, $P < 0.01$.

cortisol secretion, *i.e.* plasma cortisol concentrations and urinary free cortisol, which are relatively insensitive. Urinary free cortisol is a small fraction (<5%) of total cortisol metabolite excretion, determined principally by free plasma cortisol clearance. The sum of the urinary metabolites of cortisol in 24 h urine, as used in this study, provides a better assessment of 24 h secretion of cortisol (48). Using this method, the current study showed that cortisol secretion over 24 h is normal in lean patients with type 2 diabetes or impaired glucose tolerance.

The rate of cortisol secretion is controlled by central drive to the HPA axis and by negative feedback suppression by glucocorticoids. Dexamethasone suppression of plasma cortisol is the conventional test to examine negative feedback. Previous studies in patients with diabetes have used 1 mg of dexamethasone (34, 35, 38), as is used in clinical practice to detect Cushing's syndrome, and found that in most cases suppression was normal. Interpretation of this test is qualitative rather than quantitative, because the vast majority of controls and patients suppress to below the detection limit for plasma cortisol. As previously described (16, 17) we have selected 250 μg of dexamethasone as an approximate ED_{50} dose to quantify more subtle variations in suppression within the non-Cushing's range. Using this very low dose test, we have shown that patients with type 2 diabetes have greater sensitivity of the HPA axis to negative feedback. This could not be accounted for by differences in dexamethasone concentrations achieved. Although recent data suggest differences in the feedback response to synthetic and endogenous glucocorticoids in man (59), the finding of normal 24 h secretion in the face of this enhanced feedback sensitivity suggests that another factor is driving cortisol secretion.

Obese individuals also show increased cortisol secretion in spite of normal or increased feedback sensitivity (21). Here, increased metabolic clearance of cortisol (23), principally by 5α-reductase (14, 22) but with increased 5β-reduced metabolites also (20), may be a driving force for the increase in cortisol secretion. In this study, we found increased relative excretion of 5α- and, most strikingly, 5β-reduced cortisol metabolites in the absence of obesity in the hyperglycemic group. Notably, it has been shown that insulin therapy reduces excretion of 5α-reduced cortisol metabolites (44). This suggests that peripheral clearance of cortisol is enhanced by mechanisms directly associated with relative insulin deficiency and hyperglycemia. An alternative explanation is that inappropriate central drive to the HPA, rather than enhanced cortisol clearance, is maintaining cortisol secretion in the face of enhanced feedback in these individuals. This is consistent, for example, with the observation that habituation of cortisol in response to repeated sampling is impaired in hyperglycemic men (19).

The finding of normal cortisol secretion and circulating cortisol levels in hyperglycemic patients suggests that if cortisol is to play a role in the pathogenesis of type 2 diabetes, it will be determined by variations in peripheral tissue sensitivity to cortisol. One important determinant of tissue response to cortisol is the extent of metabolism of cortisol within the target tissues by 11β-hydroxysteroid dehydrogenases (11β-HSDs). Two enzymes exist: 11β-HSD 1, which reactivates cortisone to cortisol and serves to maintain adequate exposure of glucocorticoid receptors to cortisol (5); and 11β-HSD 2, which converts cortisol to cortisone and prevents cortisol from gaining inappropriate access to mineralocorticoid receptors. Overall activities of these enzymes can be inferred from the balance of cortisol and cortisone metabolites in urine. These have been measured in previous studies in patients with type 1 diabetes (41), in whom the ratio of cortisol/cortisone metabolites was lower than in controls, and type 2 diabetes (44), in which there was no difference between relatively obese patients and controls. However, these urinary ratios are insensitive to tissue-specific changes in 11β-HSD 1 activity (30). In obese rats (60) and humans, 11β-HSD 1 is decreased in liver (61) but increased in adipose tissue (20, 27, 28). Here, in nonobese hyperglycemic men, hepatic first pass conversion of cortisone to cortisol was impaired, albeit to a lesser extent than in obese subjects (20, 61). However, there was no change in adipose 11β-HSD 1 activity, albeit that here we biopsied sc fat from the gluteal region where previously we have biopsied from the periumbilical region (20) and that relatively few subjects (n = 17 of the original 50) returned for a biopsy. Nonetheless, there is no trend to suggest that anything approaching the approximately 3-fold differences observed in obesity occur in lean hyperglycemic subjects. The mechanism for tissue-specific dysregulation of 11β-HSD 1 in obesity is unknown (62, 63), but these data hint that hepatic dysregulation is related to insulin action, whereas adipose dysregulation is determined by some other factor associated with obesity, or indeed may be a primary mechanism in obesity (8, 64). We tested whether variations in A-ring reductase activities might explain variation in hepatic 11β-HSD 1 but did not find any correlations. It is intriguing to speculate that down-regulation of 11β-HSD 1 is a compensatory mechanism to protect the liver from glucocorticoid excess in obesity

and hyperglycemia; it may be that the lack of simultaneous increase in adipose 11β-HSD 1 explains why the group of patients studied here are members of the unusual cohort with impaired glucose tolerance without obesity. Importantly, inhibition of 11β-HSD 1 has been proposed as a therapy to improve metabolic control in diabetes and obesity (29, 64); these data suggest that sufficient 11β-HSD 1 activity exists in patients with type 2 diabetes to make this strategy worthwhile, although it remains to be seen whether inhibition in liver and/or adipose tissue will be most influential.

Another factor that is important in determining the tissue response to cortisol is the expression and activity of glucocorticoid receptors, which is difficult to measure *in vivo* in man. Studies comparing sensitivity to synthetic glucocorticoid receptor agonists in different sites suggest that there can be tissue-specific differences. For example, although sensitivity in skin correlates with that in lung (65), it may not correlate with that in leukocytes (66) or in the HPA axis (67). In this study, we show that dermal vascular sensitivity to beclomethasone dipropionate is increased in patients with glucose intolerance. Similar findings have been described in hypertensive and insulin resistant men (13, 17). This provides circumstantial evidence that glucocorticoid receptors are more readily activated in dermal vessels, although there may be confounding factors influencing the dermal blanching response. Up-regulation of glucocorticoid receptor expression has been implicated in the pathophysiology of insulin resistance in animal models (68). Moreover, glucocorticoid receptor mRNA levels in skeletal muscle are elevated in men with insulin resistance (69, 70). These observations suggest that therapeutic strategies to alter glucocorticoid action in key insulin-sensitive target tissues are likely to be especially beneficial in hyperglycemic patients.

In summary, we have demonstrated that patients with type 2 diabetes or glucose intolerance exhibit abnormalities in cortisol action in the absence of hypertension or obesity. These findings add further weight to the hypothesis that abnormalities in cortisol action may be a factor that links insulin resistance, hypertension, glucose intolerance, and obesity.

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Address all correspondence and requests for reprints to: Prof. Brian R. Walker, British Heart Foundation Senior Research Fellow, University of Edinburgh, Endocrinology Unit, Department of Medical Sciences, Western General Hospital, Edinburgh EH4 2XU, United Kingdom. E-mail: B.Walker@ed.ac.uk.

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Present address for R.C.A.: Lecturer in Medicine, University of Bristol, University Research Centre for Neuroendocrinology, Bristol Royal Infirmary, Bristol BS2 8HW, United Kingdom.

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Endothelium-derived hyperpolarizing factor and potassium use different mechanisms to induce relaxation of human subcutaneous resistance arteries

[1]C.-A. McIntyre, [1]C.H. Buckley, [1]G.C. Jones, [1]T.C. Sandeep, [1]R.C. Andrews, [2]A.I. Elliott, [3]G.A. Gray, [1]B.C. Williams, [2]J.A. McKnight, [1]B.R. Walker & *,[1]P.W.F. Hadoke

[1]Department of Medical Sciences, Western General Hospital, University of Edinburgh, Edinburgh; [2]Metabolic Unit, Western General Hospital, University of Edinburgh, Edinburgh and [3]Department of Biomedical Sciences, Hugh Robson Building, University of Edinburgh, Edinburgh

1 This investigation examined the hypothesis that release of K^+ accounts for EDHF activity by comparing relaxant responses produced by ACh and KCl in human subcutaneous resistance arteries.

2 Resistance arteries (internal diameter 244 ± 12 μm, $n=48$) from human subcutaneous fat biopsies were suspended in a wire myograph. Cumulative concentration-response curves were obtained for ACh ($10^{-9}-3\times10^{-5}$ M) and KCl (2.5–25 mM) following contraction with noradrenaline (NA; 0.1–3 μM).

3 ACh (E_{max} $99.07 \pm 9.61\%$; $-\text{LogIC}_{50}$ 7.03 ± 0.22; $n=9$) and KCl (E_{max} $74.14 \pm 5.61\%$; $-\text{LogIC}_{50}$ 2.12 ± 0.07; $n=10$)-induced relaxations were attenuated ($P<0.0001$) by removal of the endothelium (E_{max} $8.21 \pm 5.39\%$ and $11.56 \pm 8.49\%$, respectively; $n=6-7$).

4 Indomethacin (10 μM) did not alter ACh-induced relaxation whereas L-NOARG (100 μM) reduced this response (E_{max} $61.7 \pm 3.4\%$, $P<0.0001$; $n=6$). The combination of ChTx (50 nM) and apamin (30 nM) attenuated the L-NOARG-insensitive component of ACh-induced relaxation (E_{max}: $15.2 \pm 10.5\%$, $P<0.002$, $n=6$) although these arteries retained the ability to relax in response to 100 μM SIN-1 (E_{max} $127.6 \pm 13.0\%$, $n=3$). Exposure to $BaCl_2$ (30 μM) and Ouabain (1 mM) did not attenuate the L-NOARG resistant component of ACh-mediated relaxation (E_{max}, 76.09 ± 8.92, $P=0.16$; $n=5$).

5 KCl-mediated relaxation was unaffected by L-NOARG + indomethacin (E_{max}; $68.1 \pm 5.6\%$, $P=0.33$; $n=5$) or the combination of L-NOARG/indomethacin/ChTx/apamin (E_{max}; $86.61 \pm 14.02\%$, $P=0.35$; $n=6$). In contrast, the combination of L-NOARG, indomethacin, ouabain and $BaCl_2$ abolished this response (E_{max}, $5.67 \pm 2.59\%$, $P<0.0001$, $n=6$).

6 The characteristics of KCl-mediated relaxation differed from those of the nitric oxide/prostaglandin-independent component of the response to ACh, and were endothelium-dependent, indicating that K^+ does not act as an EDHF in human subcutaneous resistance arteries.

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Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; ChTx, charybdotoxin; EDHF, endothelium-derived hyperpolarizing factor; EDTA, ethylene diamine tetraacetic acid; F, Female; KPSS, high potassium physiological salt solution; L-NOARG, N^G-nitro-L-arginine; M, Male; NA, noradrenaline; NO, nitric oxide; PG, prostaglandin; PSS, physiological salt solution; SIN-1, 3′-morpholinosydnonimine

Introduction

The vascular endothelium modulates agonist-dependent relaxation by releasing substances such as nitric oxide (NO) and prostaglandins (PGs) (Furchgott & Vanhoutte, 1989). In some vessels, particularly those with a small diameter (Shimokawa *et al.*, 1996), a component of the endothelium-dependent relaxation is insensitive to nitric oxide synthase and cyclooxygenase inhibition (Nagao *et al.*, 1992; Brandes *et al.*, 1997). This component appears to be mediated by hyperpolarization of the vascular smooth muscle cells (Brayden, 1990), suggesting the existence of a distinct endothelium-derived hyperpolarizing factor (EDHF) (Taylor & Weston, 1988; Feletou & Vanhoutte, 1997).

The identity of EDHF has yet to be confirmed, although activity of this factor has been attributed to epoxyeicosatrienoic acids (Hecker *et al.*, 1994), endocannabinoids (Randall *et al.*, 1996), hydrogen peroxide (Matoba *et al.*, 2000) and the presence of myoendothelial gap junctions (Chaytor *et al.*, 1998). A recent study suggested that release of K^+ into the myoendothelial space accounted for EDHF activity in rat hepatic and mesenteric arteries (Edwards *et al.*, 1998). In this study, EDHF-mediated responses (but not those to exogenous K^+) were inhibited by using charybdotoxin (ChTx) and apamin to block large (BK_{Ca}) and small (SK_{Ca}) conductance calcium-activated potassium channels on

*Author for correspondence at: Department of Medical Sciences, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU; E-mail: phadoke@srv0.med.ed.ac.uk

the endothelium. In contrast, the combination of barium and ouabain inhibited responses to K^+ as well as to EDHF, suggesting that both EDHF and K^+ cause smooth muscle cell hyperpolarization through activation of inward rectifier potassium channels (K_{IR}) and Na^+/K^+-ATPases. Subsequent studies have, however, challenged the identification of K^+ as EDHF by demonstrating differences in the characteristics of EDHF and K^+-induced relaxation in rat mesenteric (Doughty *et al.*, 2000; Lacy *et al.*, 2000), porcine coronary and guinea-pig carotid arteries (Quignard *et al.*, 1999).

Human resistance arteries have been used extensively to examine the cardiovascular defects associated with the development of a variety of different disease processes. A large component of endothelium-dependent relaxation in these arteries is mediated by EDHF (Nakashima *et al.*, 1993; Urakami-Harasawa *et al.*, 1997; Wallerstedt & Bodelsson, 1997) but the mechanism of this response has not been elucidated. This investigation aimed to determine whether K^+ accounted for EDHF activity in human subcutaneous resistance arteries by comparing the NO-independent, PG-independent component of ACh-induced relaxation with relaxant responses produced by exogenous potassium.

Methods

Vessel preparation

Biopsies of gluteal skin and subcutaneous fat (2 cm × 1 cm × 1 cm) were obtained under local anaesthesia (2% lignocaine hydrochloride; Astra, Herts, U.K.) from 26 healthy volunteers (20 Male, six Female; age 46 ± 3 years). Written informed consent and approval from the Lothian Research Ethics Committee were obtained. Each biopsy was immersed immediately in cold (4°C) physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 24, KH_2PO_4 1.18, K_2EDTA 0.026 and D-glucose, 5.5. Dissection of these biopsies provided 48 resistance artery sections (mean internal diameter 244 ± 12 μm) for pharmacological analysis. Ring segments of these arteries, 2 mm in length, were suspended on two 40 μm stainless steel wires in a small vessel myograph for measurement of isometric force. The myograph bath contained PSS maintained at 37°C and perfused with 95% O_2/5% CO_2. Following an equilibration period of 30 min, the resting tension-internal circumference relationship was determined by stepwise radial stretching and the vessels were set to their optimum resting level (0.9 L_{100}, where L_{100} is the internal circumference the vessels would have when relaxed and subjected to a pressure of 100 mmHg; Mulvany & Halpern, 1977). After equilibration for a further 30 min, vessel viability was assessed using a standard start procedure (Aalkjaer *et al.*, 1987). This consisted of five consecutive stimulations lasting 3 min, each followed by a 5 min washout period. The first, second and fifth contractions were produced using a high (125 mM) potassium solution (KPSS; made by equimolar substitution of KCl for NaCl in PSS) containing 10 μM noradrenaline (NA). The third was obtained with NA (10 μM) alone and the fourth with KPSS alone. The functional integrity of the endothelium was assessed by adding ACh (0.1 – 10 μM) to vessels contracted with sufficient NA (0.1 – 3 μM) to produce 60 – 80% of the response KPSS.

The contribution of EDHF to ACh-mediated relaxation

Sixteen resistance arteries (internal diameter 183 ± 15 μm) from 14 male subjects (age 57 ± 12 years) were used for this part of the investigation. After the standard start procedure, a cumulative concentration-response curve to ACh (0.001 – 300 μM) was obtained following precontraction with a sub-maximal concentration (0.1 – 3 μM) of NA (to produce a contraction of ~60 – 80% the maximum response to KPSS). The artery was washed with PSS (37°C) and the procedure repeated following incubation with either; (a) indomethacin (10 μM for 45 min, $n=6$), (b) N^G-nitro-L-Arginine (L-NOARG; 100 μM for 45 min, $n=6$), or (c) L-NOARG (100 μM for 45 min), plus charybdotoxin (ChTx; 50 nM for 10 min) and apamin (30 nM for 10 min, $n=6$). Arteries were exposed to only one antagonist except for two of those initially incubated with indomethacin which were subsequently exposed to the combination L-NOARG + ChTx + apamin. Three of the arteries incubated with L-NOARG + ChTx + apamin, were also exposed to a single concentration (100 μM) of the exogenous NO donor, 3′-morpholinosydnonimine (SIN-1) once the concentration-response curve to ACh had been completed.

Comparison of K^+-induced relaxation with the EDHF-mediated component of ACh-evoked relaxation

Thirty-two resistance arteries (internal diameter 273 ± 14 μm) obtained from 12 subjects (six male, six female; age 32 ± 4 years) were used for this part of the investigation. The endothelium was removed from some arteries by rubbing the luminal surface with a single hair. Cumulative concentration-response curves were obtained using ACh (0.001 – 300 μM) and KCl (2.5 – 25 mM), in intact ($n=9-10$) and denuded ($n=6-7$) arteries, after pre-contraction (to produce a contraction of ~60 – 80% the maximum response to KPSS) with a sub-maximal concentration of NA (0.1 – 3 μM). Responses to KCl were repeated following incubation with a combination of either (a) L-NOARG (100 μM) + indomethacin (10 μM; 45 min, $n=5$); (b) L-NOARG (100 μM) + indomethacin (10 μM for 45 min) plus charybdotoxin (ChTx; 50 nM for 10 min) and apamin (30 nM for 10 min, $n=6$) or (c) L-NOARG (100 μM) + indomethacin (10 μM for 45 min) plus $BaCl_2$ (30 μM for 10 min) and ouabain (1 mM for 10 min, $n=6$). Concentration-response curves to ACh were also produced in the arteries exposed to the combinations described for groups (b) and (c).

Drugs

All salts were obtained from BDH Laboratory supplies, (Poole, Dorset, U.K). All drugs were purchased from Sigma, (Poole, Dorset, U.K.), except for 3′ morpholinosydnonimine, charybdotoxin and apamin which were obtained from Alexis Corporation Ltd (Nottingham, U.K.). Acetylcholine chloride, ouabain, barium chloride and noradrenaline bitartrate were dissolved in distilled water; indomethacin in 1.5×10^{-3} M Na_2CO_3 (final bath concentration of Na_2CO_3 did not exceed 0.015 mM) and apamin in 0.05 M acetic acid (final bath

concentration of acetic acid did not exceed 0.15 mM). Charybdotoxin was dissolved in a Tris buffer (10 mM, pH 7.5) containing 0.1% BSA, 100 mM NaCl and 1 mM EDTA (final bath concentrations of NaCl and EDTA did not exceed 5 and 0.05 mM, respectively). 0.01% BSA was added to the myograph chamber before applying the toxins. Stock solutions were stored at −20°C, thawed as required and subsequent dilutions made in distilled water. The concentrations quoted are final molar concentrations in the organ bath.

Statistics

All values are presented as mean±standard error mean (s.e.mean) from n experiments (where n represents the number of subjects). Relaxation responses to ACh and KCl are expressed as a percentage of the initial NA-induced precontraction. The concentration of agonist required to produce 50% of the maximum response (IC_{50}) was obtained by fitting the Hill equation to the data using curve fitting software (Fig. P, Biosoft, Cambridge, U.K.) and is expressed as the negative logarithm of the IC_{50} ($-\log IC_{50}$). Comparisons of maximum relaxation and $-\log IC_{50}$ values were made using Student's paired or unpaired t-test, as appropriate, and significance was assumed when $P<0.05$.

Results

The contribution of EDHF to ACh-induced relaxation

ACh caused approximately 80–100% relaxation in intact human subcutaneous resistance arteries following pre-contraction with a sub-maximal concentration of NA (0.1–3 μM; Figure 1). None of the inhibitors caused an increase in either the resting tone of the arteries or the response to the pre-contracting concentration of NA.

Incubation with indomethacin (Figure 1a) did not alter the magnitude (E_{max}, 97.56±1.83%, $n=6$) or sensitivity ($-\log IC_{50}$, 7.24±0.20, $n=6$) of ACh-evoked relaxation when compared with controls (90.80±4.69%, $P=0.18$ and 7.23±0.25 $P=0.96$, respectively; $n=6$). In contrast, exposure to L-NOARG (Figure 1b) resulted in a significant ($P<0.0001$), although not total, reduction in maximum relaxation (61.68±3.38%, $n=6$) compared with controls (91.55±3.95%, $n=6$) with a corresponding reduction in sensitivity ($-\log IC_{50}$, 6.41±0.10 vs 7.19±0.13, respectively, $P<0.005$; $n=6$). Arteries exposed to the combination of L-NOARG plus ChTx and apamin demonstrated almost total attenuation of ACh-mediated relaxation (E_{max}, 15.2±10.5%, $n=6$) despite producing a full concentration-response curve before exposure to these inhibitors (E_{max}, 92.59±3.65%, $P<0.002$; $-\log IC_{50}$, 7.70±0.30, $n=6$). These arteries maintained their ability to relax in response to exogenous NO, as SIN-1 (100 μM) caused complete relaxation in the presence of L-NOARG, ChTx and apamin (127.6±13.0%; $n=3$).

Comparison of K^+-induced relaxation with the EDHF-mediated component of ACh-evoked relaxation

Relaxation responses were obtained using potassium in 10 arteries with an intact endothelium and responses to ACh were also tested in nine of these. Typical relaxation responses were obtained with ACh (E_{max}, 99.07±9.61%; $-\log IC_{50}$, 7.03±0.224; $n=9$), which produced a sustained concentration-dependent relaxation (Figure 2). In contrast, although

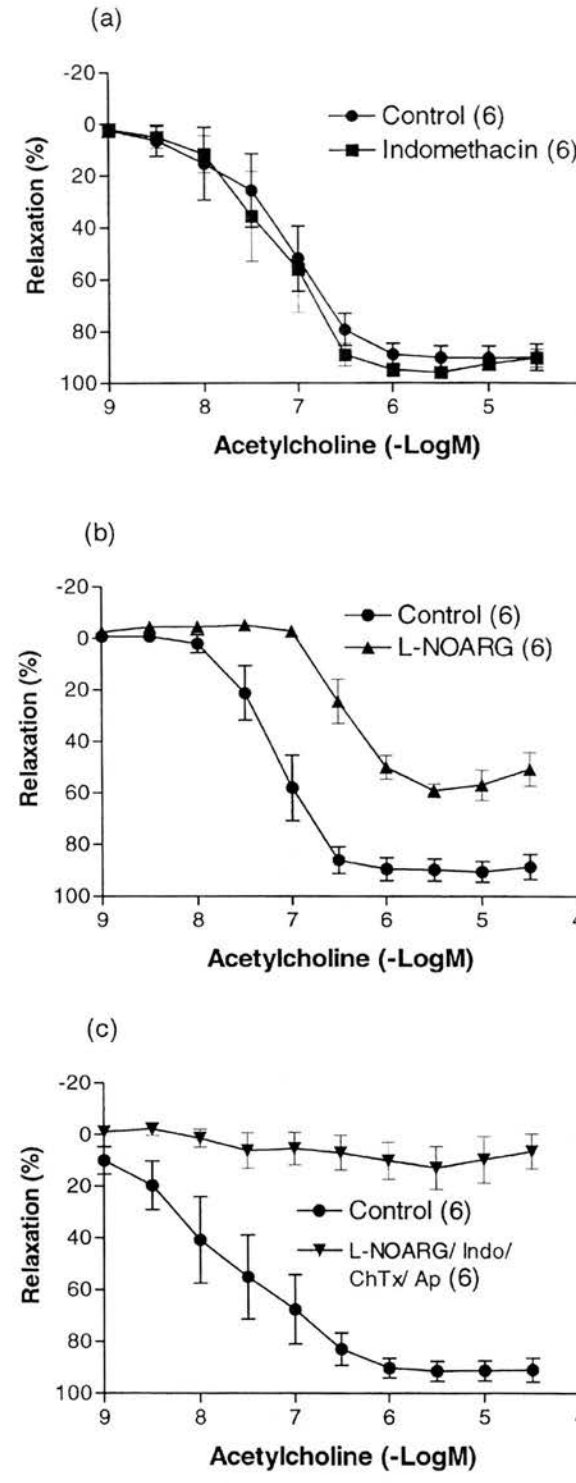

Figure 1 Cumulative concentration-response curves to ACh (10^{-9}–3×10^{-5} M) before and after incubation with the following combination of inhbitors (a) the cyclooxygenase inhibitor indomethacin (10 μM for 45 min), (b) the NO synthase inhibitor L-NOARG (100 μM for 45 min) or (c) L-NOARG (100 μM for 45 min) plus the K^+ channel blockers ChTx (50 nM for 10 min) and apamin (30 nM for 10 min). Results are shown as mean±s.e.mean, for (n) arteries.

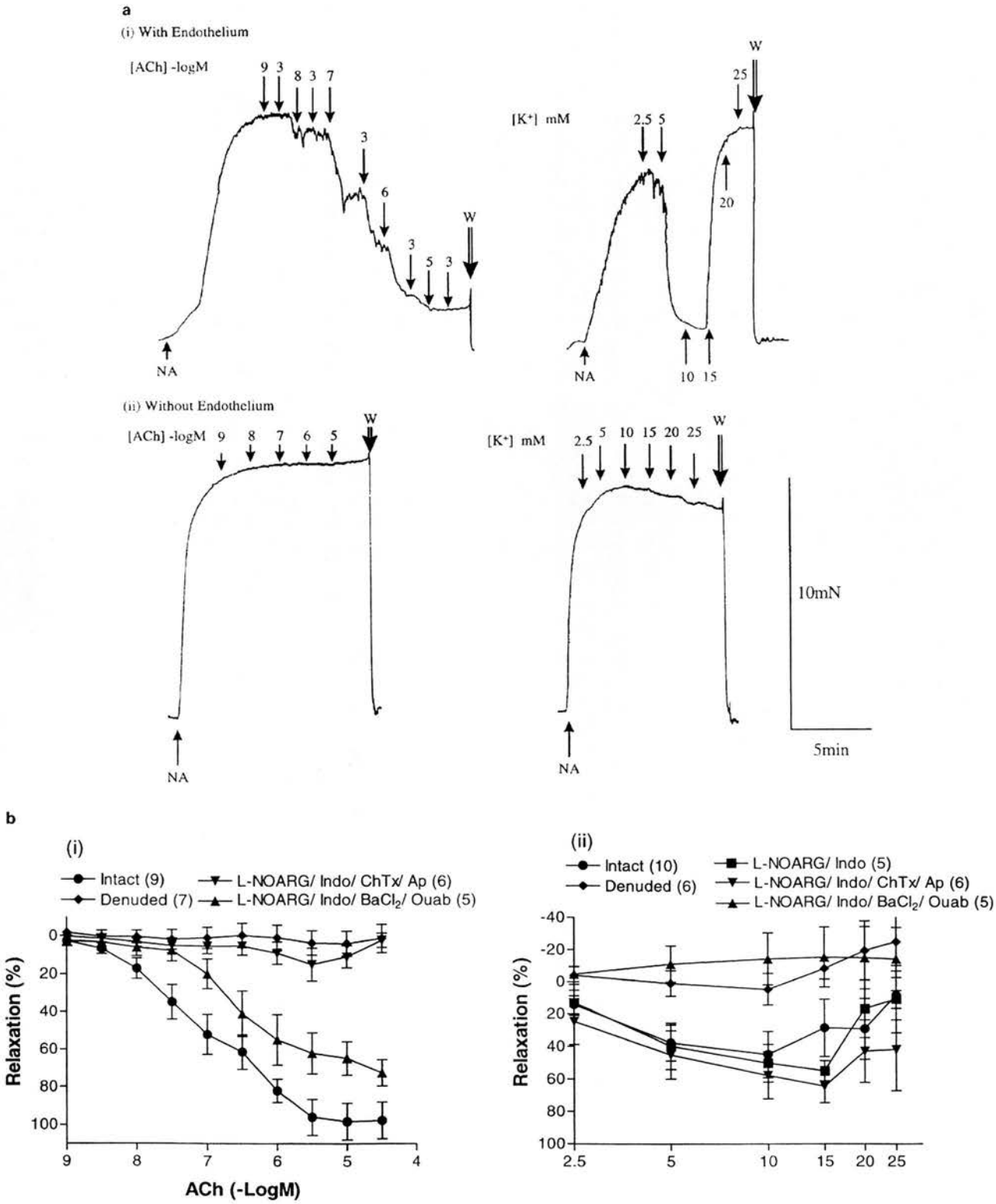

Figure 2 Comparison of ACh- and KCl-mediated relaxation. (a) Representative traces showing (i) relaxation responses of an intact artery to acetylcholine and KCl and (ii) the effect of removal of the endothelium on these responses. (b) Cumulative concentration-response curves for (i) acetylcholine and (ii) KCl obtained in arteries with and without an intact endothelium or in the presence of L-NOARG (100 μM) and indomethacin (10 μM) alone or combined with either ChTx (50 nM) and apamin (30 nM) or $BaCl_2$ (30 μM) plus ouabain (1 mM). Results are shown as mean $\pm$ s.e.mean, for (n) arteries.

potassium also relaxed these resistance arteries (E_{max}, 74.14±5.61%, IC_{50}, 6.09±1.17 mM; $-\log IC_{50}$, 2.12±0.07, $n=10$), the response to this compound (Figure 2) was inconsistent and was superseded by a reversal of the initial relaxation response as the concentration of KCl rose (>15–25 mM). As expected, removal of the endothelium virtually abolished responses to ACh (13.34±6.16%, $n=7$, $P<0.0001$) but also abolished potassium-mediated relaxation (E_{max}, 15.53±9.18%, $n=6$, $P<0.001$) (Figure 2).

The potassium-induced relaxation was not affected by incubation with L-NOARG and indomethacin (E_{max}, 68.1±5.6%, $P=0.51$; IC_{50}, 5.74±1.86 mM; $-\log IC_{50}$, 2.34±0.15, $P=0.33$, $n=5$) or with the combination of L-NOARG with indomethacin, ChTx and apamin (E_{max}, 86.61±14.02%; $P=0.35$; IC_{50}, 6.78±2.90 mM; $-\log IC_{50}$, 2.68±0.52, $P=0.23$, $n=6$). Indeed the maximum relaxation evoked by potassium tended to be larger in the latter group. Exposure of vessels to the combination of $BaCl_2$ and ouabain resulted in an increase in basal tone of 0.40±0.17 mN (equivalent to 16.6±7.4% of the maximum response to KPSS; $n=11$). This tended to be larger in arteries used for producing responses to ACh (22.4±17.6% KPSS; $n=5$) than in those subsequently exposed to KCl (12.0±5.6% KPSS; $n=6$). Once this contraction had stabilized, vessels were contracted with sufficient NA (0.1–3 μM) to produce a contraction 60–80% the size of the maximum response to KPSS (responses to ACh obtained in one artery were discarded as the combination of $BaCl_2$ plus ouabain produced a contraction equivalent to 80% of the response to KPSS). Potassium-induced relaxation was totally abolished by incubation with the combination of L-NOARG with indomethacin, $BaCl_2$ and ouabain (5.7±2.6%; $n=6$; $P<0.0001$). In contrast, a considerable ACh-induced relaxation remained evident following exposure to this combination of inhibitors although there was a trend towards reduced relaxation that did not achieve significance (E_{max}, 76.09±8.92%; $P=0.16$; $-\log IC_{50}$, 6.47±0.23; $P=0.11$, $n=5$).

Discussion

Previous investigations have demonstrated that an NO/PG-independent component of ACh-evoked relaxation is mediated by EDHF (Nakashima *et al.*, 1993; Urakami-Harasawa *et al.*, 1997; Wallerstedt & Bodelsson, 1997). Studies in arteries from experimental animals have suggested that K^+ accounts for EDHF activity (Edwards *et al.*, 1998). In order to clarify whether K^+ acts as an EDHF in human arteries, this investigation compared potassium-induced and EDHF-induced relaxation responses in subcutaneous resistance arteries isolated from biopsies of gluteal fat. The characteristics of potassium-induced relaxation were different from the EDHF-mediated response and, of significance, were abolished by removal of the endothelium. Taken together, this suggests that release of endothelium-derived K^+ into the myoendothelial space does not account for EDHF activity in human subcutaneous resistance arteries.

Comparison with previous investigations indicates that the ChTx/ apamin-sensitive, NO-independent component of ACh-evoked relaxation is mediated by EDHF. In rat mesenteric arteries contracted with an α-adrenoceptor agonist, the NO-independent component of ACh-mediated relaxation was caused by smooth muscle cell hyperpolarisation (Plane & Garland, 1996). This response is abolished by the combination of ChTx and apamin (Zygmunt & Högestätt, 1996), probably by inhibition of BK_{Ca} and SK_{Ca} on the endothelium (Doughty *et al.*, 1999). The persistence of a significant NO-independent (EDHF-mediated) relaxation in response to ACh is consistent with previous studies of human subcutaneous (Woolfson & Poston, 1990; Deng *et al.*, 1995; Hillier *et al.*, 1998), omental (Ohlmann *et al.*, 1997), gastroepiploic (Urakami-Harasawa *et al.*, 1997), coronary (Nakashima *et al.*, 1993) and pial (Petersson *et al.*, 1995) arteries. Incomplete inhibition is unlikely to account for residual relaxation as a lower concentration of L-NOARG (3×10^{-5} M) abolished ACh-induced, endothelium-dependent relaxation in the rat aorta, pulmonary and iliac arteries (Nagao *et al.*, 1992). Furthermore, incomplete inhibition of ACh-mediated relaxation was not overcome by increasing the concentration of L-NOARG (100–300 μM; Brandes *et al.*, 1997) or by the combined application of two different L-arginine analogues (Plane & Garland, 1996; Plane *et al.*, 1997). The failure of indomethacin to attenuate ACh-mediated relaxation in the present study confirms that prostanoids do not contribute to this response in the human gluteal, subcutaneous resistance artery. This is also consistent with previous studies, in our own and other laboratories, in which indomethacin was shown to have no effect on ACh-or bradykinin-mediated relaxation of human gluteal resistance arteries when applied alone or in combination with NO synthase inhibitors (Hillier *et al.*, 1998; Buckley *et al.*, 1999). The mechanism of endothelium-dependent relaxation of human resistance arteries may depend upon the origin of a particular vessel, however, as bradykinin-mediated relaxation of human omental arteries has an indomethacin-sensitive component which becomes evident in the presence of an NO inhibitor (Ohlmann *et al.*, 1997).

The ability of exogenous potassium to relax human gluteal resistance arteries compares with results obtained in resistance arteries from experimental animals (Edwards *et al.*, 1998); Quignard *et al.*, 1999; Doughty *et al.*, 2000; Lacy *et al.*, 2000). The identification of K^+ as an EDHF in the earlier study was based on a comparison with the NO/PG-independent component of the response to ACh (Edwards *et al.*, 1998); responses to both ACh and exogenous K^+ were abolished by inhibition of K_{IR} and Na^+/K^+ ATPase, indicating a common mechanism. Exogenous K^+, however, produced an endothelium-independent hyperpolarization of smooth muscle cells that was unaffected by the combination of ChTx and apamin. This is consistent with ACh stimulating release of K^+ from endothelial cells *via* ChTx/ apamin-sensitive channels. In the present study, however, the characteristics of potassium-induced and EDHF-mediated relaxation were different: whereas the ACh-induced relaxation was highly reproducible and sustained, relaxation responses to potassium were more variable and reversed readily at higher K^+ concentrations. This is consistent with a recent study showing that exogenous K^+ will only produce a reproducible, sustained relaxation of rat resistance arteries if they are bathed in a Kreb's solution lacking K^+ ions (Lacy *et al.*, 2000). More striking, however, was the demonstration that, as in the rat mesenteric (Lacy *et al.*, 2000) and renal (Jiang & Dusting, 2001) arteries, potassium-mediated relaxa-

tion of human subcutaneous arteries was abolished by removal of the endothelium. This indicates an obligatory role for the endothelium in K^+-mediated relaxation, suggesting that it may be mediated by a further endothelium-derived factor or is dependent upon myoendothelial gap junctions (Doughty *et al.*, 2000). Finally, the inability of barium and ouabain to inhibit ACh-mediated relaxation, whilst abolishing responses to potassium, indicated that these compounds caused relaxation *via* different mechanisms. This observation contrasts with the study by Edwards *et al.* (1998) but is consistent with data obtained in subsequent investigations (Quignard *et al.*, 1999; Lacy *et al.*, 2000). The ability of K^+ to relax only a proportion (~30%) of rat mesenteric resistance arteries in one study (Doughty *et al.*, 2000) suggests that this response may even vary in different regions of the same artery, possibly reflecting variations in K_{IR} and Na^+/K^+-ATPase activity (Albarwani *et al.*, 1995).

In conclusion, this investigation demonstrated an NO/PG-independent response to ACh in human subcutaeous arteries which had characteristics consistent with EDHF-mediated relaxation. The failure of exogenous potassium to produce an endothelium-independent relaxation which mimicked this response indicates that potassium is not an EDHF in these vessels.

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REVIEW

Glucocorticoids and insulin resistance: old hormones, new targets

Robert C. ANDREWS and Brian R. WALKER
University of Edinburgh, Department of Medical Sciences, Western General Hospital, Edinburgh EH4 2XU, Scotland, U.K.

ABSTRACT

Insulin resistance has been proposed as a mediator of the association between risk factors for cardiovascular disease in the population. The clinical syndrome of glucocorticoid excess (Cushing's syndrome) is associated with glucose intolerance, obesity and hypertension. By opposing the actions of insulin, glucocorticoids could contribute to insulin resistance and its association with other cardiovascular risk factors. In this review, we describe briefly the known mechanisms of insulin resistance and highlight the potential mechanisms for the effect of glucocorticoids. We then discuss factors which modulate the influence of glucocorticoids on insulin sensitivity; this highlights a novel therapeutic strategy to manipulate glucocorticoid action which may prove to be a useful tool in treating subjects with insulin resistance. Finally, we describe evidence from human studies that glucocorticoids make an important contribution to the pathophysiology of insulin resistance in the population.

INTRODUCTION

Risk factors for cardiovascular disease include hypertension, glucose intolerance and dyslipidaemia. There is evidence that these abnormalities are associated with each other more frequently than expected by chance: this cluster has been referred to as 'Reaven's Syndrome X' [1], or the 'Metabolic Syndrome'. In the last decade, further associations with this syndrome have been described, including low birthweight [2,3], central obesity [4], abnormalities of thrombosis and fibrinolysis, impaired endothelium-dependent vasodilatation [5], reproductive dysfunction in women [6] and insulin resistance. Insulin resistance can be defined as impaired sensitivity to the effects of insulin on carbohydrate metabolism. The pathophysiology of the metabolic syndrome remains poorly understood, but many have suggested mechanisms whereby insulin resistance could underlie the association between all other features. Indeed, some go as far as referring to this cluster of abnormalities as the 'Insulin Resistance Syndrome'.

Glucocorticoid hormones (mainly cortisol in man; corticosterone in rodents) are produced in the adrenal cortex under the control of the hypothalamic–pituitary–adrenal axis. They play a key role in regulating salt and water metabolism, blood pressure, immune function and metabolism. In essence, glucocorticoids are most important at times of stress, when they provide a longer-term signal to damp many of the acute responses to illness and 're-set' metabolism in favour of providing substrates for oxidative metabolism. The importance of glucocorticoids is exemplified in clinical syndromes of deficiency (Addison's disease or hypopituitarism) and excess (Cushing's syndrome). Cortisol deficiency is characterized by postural hypotension, weight loss and hypoglycaemia; cortisol excess is characterized by hypertension, central obesity and glucose intolerance. Part of the mechanism for these effects of cortisol depends on opposing the actions of insulin, i.e. inducing a state of insulin resistance.

In this review, we describe briefly the known mechanisms of insulin resistance and highlight the potential

Key words: adipose tissue, glucocorticoid hormones, glucocorticoid receptors, gluconeogenesis, hydroxysteroid dehydrogenases, insulin.

Abbreviations: ACTH, adrenocorticotrophic hormone; 11β-HSD, 11β-hydroxysteroid dehydrogenase; CBG, cortisol binding globulin; PEP-CK, phosphoenolpyruvate carboxykinase.

Correspondence: Dr Brian R. Walker.

relevance of glucocorticoids. We then discuss factors which modulate the influence of glucocorticoids on insulin sensitivity; this highlights a novel therapeutic strategy to manipulate glucocorticoid action which may prove useful in treating subjects with insulin resistance. Finally, we describe evidence from human studies that glucocorticoids make an important contribution to the pathophysiology of insulin resistance in subjects with the metabolic syndrome.

HOW DO GLUCOCORTICOIDS INDUCE INSULIN RESISTANCE?

Insulin is synthesized and released from pancreatic β-cells in response to elevations in plasma glucose concentrations, specific amino acids (e.g. arginine), potassium and parasympathetic nervous system tone. It acts on a cell-surface receptor comprising two α and two β subunits which signal through phosphorylation of insulin receptor substrate proteins. Its actions can be divided into regulation of long-term growth and short-term metabolism; these are mediated by different intracellular second messenger signalling pathways [7].

The term 'insulin resistance' is usually used to refer to the acute regulation of carbohydrate metabolism by insulin. It has been quantified by numerous methods, usually involving measurement of the plasma insulin concentration relative to plasma glucose concentration, or the amount of glucose infused to maintain euglycaemia at a fixed insulin concentration [8]. Insulin resistance may reflect impaired insulin-dependent down-regulation of hepatic glucose release and/or impaired insulin-mediated increase in peripheral glucose uptake. Which of these variables is most important in the metabolic syndrome remains controversial, and there is probably a contribution from each. Enhanced hepatic glucose release may be most important in subjects with glucose intolerance [9] whereas impaired peripheral glucose uptake may be the major defect in subjects with normal glucose tolerance [10]. In patients with severe insulin resistance, more than 50 mutations of the insulin receptor and three mutations of the insulin receptor substrate-1 protein have been characterized [7,11]. However, these mutations are rare and do not explain insulin resistance in the vast majority of patients.

Glucocorticoids are so named because it was recognized long ago that one of their actions is on carbohydrate metabolism [12]. In addition to the insulin resistance that characterizes Cushing's syndrome, manipulation of cortisol levels within the physiological range also alters insulin sensitivity in man [13]. Although subject to the limitations of measurement of hepatic glucose output in man, the effect of glucocorticoids *in vivo* appears to include both impaired insulin-dependent glucose uptake in the periphery and enhanced gluconeogenesis in the liver [14,15]. In addition, glucocorticoids oppose other actions of insulin, including its effect to reduce central appetite [16]. Numerous effects of glucocorticoids have been demonstrated *in vitro* that could contribute to these effects on carbohydrate metabolism. These are summarized below.

In addition to their effects on insulin sensitivity, glucocorticoids inhibit insulin secretion from pancreatic β-cells [17–19]. On the other hand, central actions of glucocorticoids may enhance vagal stimulation of insulin secretion [20]. The balance of these effects may be important in determining whether insulin resistance is accompanied by compensatory hyperinsulinaemia or hyperglycaemia, and may explain in part why only some patients with Cushing's syndrome develop glucose intolerance [21].

Generalized abnormalities in target organ responses to insulin

Numerous studies have examined whether glucocorticoids have a global effect to inhibit insulin receptor binding or second messenger signalling but no consensus has emerged. Studies in man have found that glucocorticoids can decrease insulin receptor binding affinity without decreasing insulin receptor numbers [22,23], decrease receptor number and affinity [24], have no effect on receptor affinity or number [25] or increase receptor number without affecting affinity [15]. Furthermore, where *in vivo* and *in vitro* studies have been carried out simultaneously, they have not been in agreement [23,26]. It seems reasonable to conclude that the small changes in insulin receptor number or binding affinity are not sufficient to explain the degree of insulin resistance seen with glucocorticoids. Moreover, the discrepancy between *in vitro* and *in vivo* observations is likely to reflect difficulty in controlling for indirect, potentially compensatory, effects of glucocorticoids. For example, very few experiments have controlled for the hyperinsulinaemia induced by glucocorticoids. When compensatory hyperinsulinaemia was prevented by streptozotocin treatment in rats, glucocorticoid-induced changes in insulin receptor number, insulin receptor substrate-1 (IRS-1) and phosphorylation were abolished [27].

Tissue-specific determinants of insulin response

The effect of insulin receptor activation differs between tissues, since it depends primarily on altered activity of glucose transporters in peripheral tissues such as fat and skeletal muscle and enzymes influencing glycogen storage, glycolysis and gluconeogenesis in the liver. The effects of glucocorticoids on these pathways are illustrated in Figures 1 and 2.

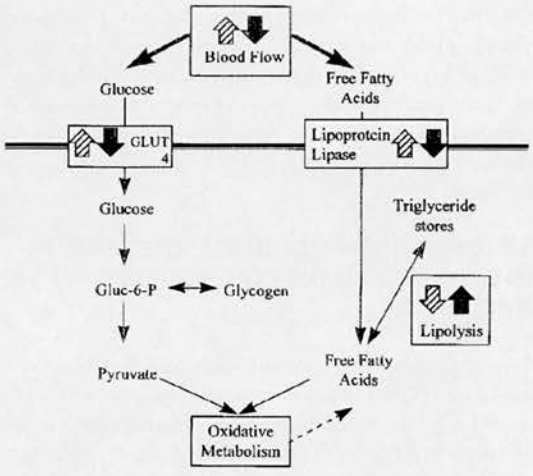

Figure 1 Effects of glucocorticoids on peripheral glucose uptake

A schematic for an archetypal insulin-sensitive cell is shown. In adipocytes, lipogenic pathways predominate whereas in skeletal muscle either oxidative metabolism (of pyruvate or free fatty acids) or glycogen synthesis predominates. GLUT 4 is expressed principally in skeletal muscle and lipoprotein lipase principally in adipose tissue. Actions of glucocorticoids (grey arrows) and insulin (striped arrows) are shown either as positive (arrow up) or negative (arrow down) effects. The major effects of glucocorticoids may be to reduce insulin-mediated vasodilatation, reduce translocation of GLUT 4 to the cell surface and enhance lipolysis, perhaps by inducing local synthesis of adrenaline (see text), thereby increasing free fatty acid competition with pyruvate for mitochondrial oxidative metabolism.

Metabolic determinants of peripheral glucose uptake

The first determinant of insulin-dependent peripheral glucose uptake is the availability on the cell membrane of the GLUT 4 transporter, which is expressed mainly in skeletal muscle and is increased by insulin. To date, mutations in GLUT 4 have not been associated with insulin resistance [28]. The expression of GLUT 4 is, in fact, increased by glucocorticoids in skeletal muscle and adipose tissue. However, translocation of GLUT 4 to the cell surface in response to insulin and to other stimuli (e.g. hypoxia) is inhibited in the presence of glucocorticoids [29–33].

The rate of glucose transport also depends on the gradient of glucose concentration across the cell membrane. This is influenced both by local delivery of glucose, determined in euglycaemic conditions by blood flow, and by the rate of removal of glucose by oxidation of pyruvate inside the cell membrane (Figure 1). Oxidation of pyruvate is influenced by competing substrates including non-esterified free fatty acids. Acute administration of free fatty acids results in insulin resistance [34]. Free fatty acids are increased in some subjects with the metabolic syndrome, especially those who are obese. Acipimox and nicotinic acid, which lower free fatty acid concentrations, also increase insulin sensitivity [35]. However, chronic administration of free fatty acids does not induce insulin resistance [36], and free fatty acids may be elevated in insulin-resistant subjects because of impaired insulin-dependent down-regulation of lipolysis. Thus, as for other associations of insulin resistance, elevated free fatty acids could both result from, and contribute to, impaired insulin-dependent glucose uptake.

Increased lipolysis may be important in glucocorticoid-induced insulin resistance, since this is reversed by inhibiting lipolysis [37] or lipid oxidation [38] (Figure 1). Again, however, cause and effect are difficult to elucidate because free fatty acids have been reported to influence glucocorticoid receptor binding [39,40]. Increased lipolysis induced by glucocorticoids may be mediated indirectly, by up-regulation of phenylethanolamine *N*-methyltransferase [41], an enzyme expressed in skeletal muscle that converts noradrenaline into adrenaline. Inhibition of phenylethanolamine *N*-methyltransferase ameliorates glucocorticoid-induced insulin resistance [41]. Alternatively, effects on lipolysis may be mediated via up-regulation of peroxisome-proliferator-activated γ receptors, for which the insulin-sensitizing thiazolidinediones are exogenous ligands but endogenous ligands have yet to be identified [42]. Finally, glucocorticoids may increase circulating free fatty acids by inhibiting lipoprotein lipase (Figure 1) [43].

Non-metabolic determinants of peripheral glucose uptake

Recent work by Baron et al. [44] has demonstrated that insulin induces endothelium-dependent vasodilatation, probably mediated by increased nitric oxide synthesis or action. It has been suggested that this action contributes to enhanced glucose uptake in response to insulin and other vasodilator stimuli, particularly in skeletal muscle. Moreover, this model suggests that the impaired endothelium-dependent vasodilatation in subjects with features of the metabolic syndrome (hypercholesterolaemia [45], hypertension [46] or diabetes mellitus) could both result from, and contribute to, impaired insulin action in skeletal muscle. However, others have found that increased blood flow and glucose uptake during hyperinsulinaemia are dissociated in man [47].

Glucocorticoids may also influence this determinant of insulin sensitivity. We have recently shown that glucocorticoids impair endothelium-dependent vasodilatation in humans *in vivo* ([48]; G. Mangos, B. Walker, J. Kelly, D. Webb and J. Whitworth, unpublished work) and therefore, if this is an important mechanism dictating glucose delivery, it may also be a site where insulin action is counterbalanced by glucocorticoids (Figure 1).

Hepatic glucose release

The pathways determining the balance between glycogen synthesis and glucose oxidation versus glycogenolysis

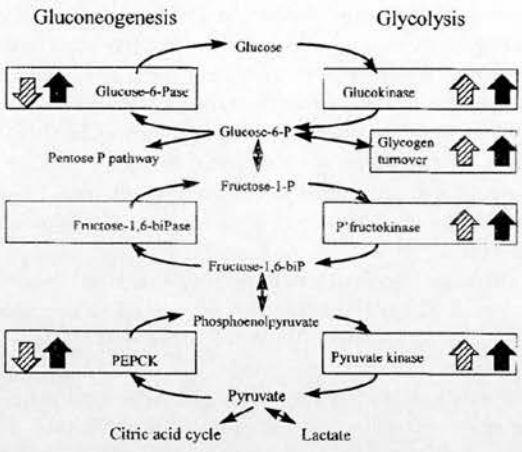

Figure 2 Effects of glucocorticoids on hepatic glucose metabolism

The principal metabolic fates of glucose in the liver are shown. Actions of glucocorticoids (grey arrows) and insulin (striped arrows) are shown either as positive (arrow up) or negative (arrow down) effects. In some respects, insulin and glucocorticoids oppose each other's actions, particularly on gluconeogenesis (PEP-CK) and release of glucose from glucose 6-phosphate. In other respects, however, insulin and glucocorticoids do not oppose each other, especially in promoting oxidative glycolysis and increasing turnover between glucose 6-phosphate and glycogen.

and gluconeogenesis are summarized in Figure 2. Abnormalities in hepatic glucose release are most likely to be manifest as increased fasting plasma glucose, as observed in Type II diabetes mellitus but not always in association with other features of the metabolic syndrome. Until very recent advances in stable isotope methodology, it was more difficult to measure hepatic than peripheral glucose metabolism in man and the mechanisms of hepatic insulin resistance remain obscure. One element of insulin signalling which may be specific to the liver, and which has not been accounted for in previous human studies, is the importance of insulin pulsatility [49]. Like other peptide receptors, the insulin receptor responds to specific patterns of change in insulin concentration as well as to the absolute level. The pulsatile pattern of insulin release is altered at an early stage in dysfunction of the pancreatic β-cell.

In the liver, contrasting effects of insulin and glucocorticoids are well-characterized in animal models [12] (Figure 2). A key effect appears to be the counter-regulation by insulin and glucocorticoids of the rate-limiting enzyme in gluconeogenesis, phosphoenolpyruvate carboxykinase (PEP-CK) [50]. However, there is a conflicting literature concerning the effects of glucocorticoids on hepatic glucose metabolism in man, which has been reported to be increased [14,25] or not affected [51,52]. These differences may reflect the difficulties of measurement in man, rather than any true discrepancy between species. Specifically, they could be accounted for by increased glucose/glucose 6-phosphate cycling, which confounds many of the tracer measurements of hepatic glucose output. Glucocorticoid effects on insulin pulsatility have yet to be reported, although as described above, glucocorticoids do influence β-cell function.

FACTORS WHICH MODULATE THE EFFECT OF GLUCOCORTICOIDS ON INSULIN SENSITIVITY

Having described the numerous potential sites of action of glucocorticoids on insulin sensitivity, we will now address the importance of altered glucocorticoid action in insulin resistance. This requires an understanding of the factors which modulate glucocorticoid action, which are shown in Figure 3.

Plasma cortisol concentrations

An important determinant of glucocorticoid action is the circulating concentration of cortisol. This is influenced both by the rate of cortisol secretion from the adrenal cortex, controlled principally by adrenocorticotrophic hormone (ACTH), and by the metabolic clearance rate of cortisol. Cortisol circulates in plasma in three states: 5–10% circulates unbound, being 'free' to cross cell membranes and interact with receptors; 70–75% is bound to corticosteroid binding globulin (CBG); and 15–20% is bound to albumin. CBG and albumin therefore act to buffer the free cortisol concentration, but these are saturated within the high physiological range so that there are large excursions in free plasma cortisol concentrations between peaks (in the morning in man and during stress) and troughs (at night in man).

Tissue sensitivity to cortisol

In addition to the influence of changes in circulating cortisol levels, the last decade has seen the recognition of the importance of tissue-specific variations in the mechanisms dictating target organ sensitivity to glucocorticoids. Cortisol can activate either glucocorticoid (type 2 corticosteroid) or mineralocorticoid (type 1 corticosteroid) receptors, and indeed has higher affinity for the latter [53]. Glucocorticoid receptors are more widely distributed and act as high-capacity, low-affinity receptors that are occupied mostly during the circadian peak of plasma cortisol levels in the morning in man. In contrast, mineralocorticoid receptors have a more restricted localization. At some sites, e.g. in hippocampus and hypothalamus, they act as low-capacity, high-affinity receptors that are fully occupied by cortisol during the circadian peak, but variably occupied during the nocturnal trough of cortisol secretion in man, and may be involved in negative-feedback control of the hypothalamic–pituitary–adrenal axis [54]. At other sites, e.g. in

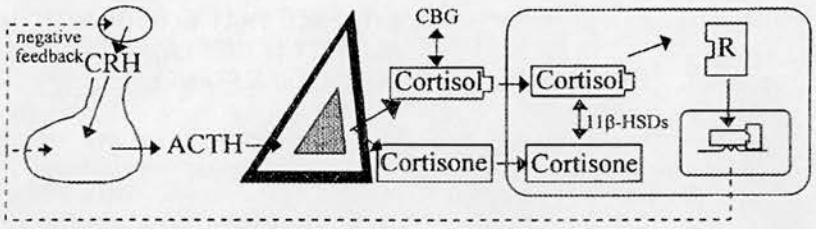

Figure 3 Factors determining glucocorticoid action

Schematic indicates the hypothalamicñpi tuitaryñadrenal axis controlling secretion of both active glucocorticoid (cortisol) and inactive cortisone. These steroids circulate in similar free concentrations although free cortisol is in equilibrium with a pool of cortisol bound to CBG and albumin. Also shown is a schematic target cell, in which interconversion of cortisol and cortisone by 11β-HSDs dictates access of glucocorticoid to receptors (R) and subsequent regulation of target genes, including those responsible for negative feedback. CRH, corticotrophin-releasing hormone.

distal nephron, sweat glands and colon, they do not bind cortisol and act as receptors for the much lower plasma concentrations of aldosterone, thereby regulating salt balance [55].

For some time, it was a paradox that mineralocorticoid receptors could bind cortisol at some sites but not at others. This paradox was explained by the activity at aldosterone target sites of an enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which inactivates cortisol by converting it into the metabolite cortisone. When this mechanism is defective, as in a rare congenital syndrome of 11β-HSD2 mutations [56], or after administration of the 11β-HSD inhibitor liquorice [57], then cortisol gains inappropriate access to mineralocorticoid receptors and induces sodium retention, hypokalaemia and hypertension [58,59].

This model of enzyme-mediated regulation of ligand access to intracellular receptors is not unique to mineralocorticoid receptors. For example, thyroxine is also activated in target tissues to tri-iodothyronine by 5′-monodeiodinases, testosterone is activated by 5α-reductase to dihydrotestosterone, and similar mechanisms influence activation of vitamin D and retinoid receptors [60]. Very recently it has emerged that the access of cortisol to glucocorticoid receptors is also regulated by an enzyme, and that this is relevant to the effects of cortisol on insulin sensitivity.

Modulation of insulin sensitivity by 11β-HSD type 1

Before the cloning of 11β-HSD2 [61,62], which catalyses the inactivation of cortisol to cortisone, a different isoenzyme (11β-HSD type 1; 11β-HSD1) had been cloned [63]. 11β-HSD1 catalyses the same dehydrogenase reaction in solution *in vitro*, but is now recognized to function predominantly as a reductase, re-activating cortisone to cortisol in many tissues, including in whole cells in culture [64–68], in perfused organs [69] and *in vivo* in man [70]. 11β-HSD1 is widely distributed, including in liver, adipose tissue and skeletal muscle. We hypothesize that its function in liver is to ensure adequate

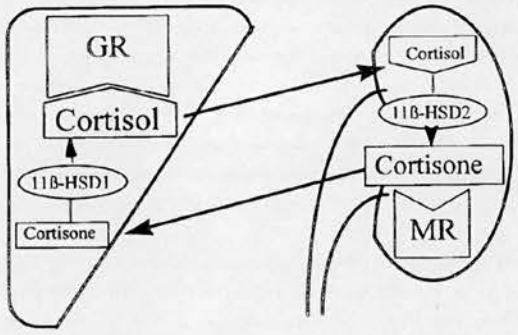

Figure 4 Contrasting influence of 11β-HSDs on cortisol sensitivity in liver and kidney

Predominant conversion of cortisol into cortisone by the dehydrogenase 11β-HSD2 in kidney results in protection of local mineralocorticoid receptors (MR). Predominant conversion of cortisone into cortisol by the reductase 11β-HSD1 in liver results in enhanced activation of glucocorticoid receptors (GR).

activation of low-affinity glucocorticoid receptors, by re-activating cortisone into cortisol (Figure 4). The evidence given below supports this hypothesis.

1. Circulating levels of cortisone in man are approximately 50 nM, and are not protein bound or subject to circadian variation [70]. This compares with free plasma cortisol concentrations of 50–100 nM in the morning and approximately 10 nM in the evening. There is therefore an ample supply of substrate cortisone for re-activation to cortisol by 11β-HSD1.

2. The ratio of cortisol/cortisone in human hepatic vein is approximately five-fold higher than in arterial plasma [70], confirming that 11β-HSD1 functions as a reductase in human liver. Similarly, administration of cortisone by mouth, which is delivered to the liver by the portal circulation, results in high circulating cortisol concentrations but negligible circulating cortisone concentrations [71].

3. Administration of the liquorice derivative, carbenoxolone, inhibits the conversion of cortisone into cortisol in man [71] and also inhibits hepatic 11β-HSD1 activity in isolated perfused rat liver [69]. Carbenoxolone also

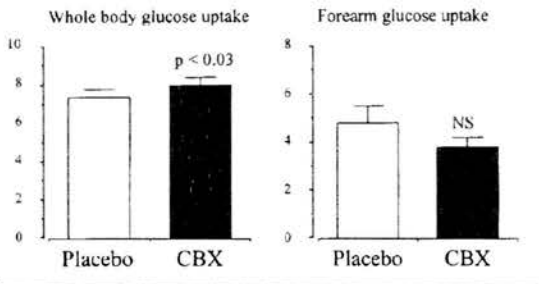

Figure 5 Effect of carbenoxolone on insulin sensitivity

Seven healthy males participated in a double-blind cross-over study comparing carbenoxolone (CBX: 100 mg 8 hourly for 8 days) with placebo. Euglycaemic hyperinsulinaemic clamps were performed with measurement of forearm glucose uptake by plethysmography and collection of arterialized and deep forearm vein samples. Whole-body insulin sensitivity is represented by the M value (in μmol · min^{-1} · kg^{-1}), or rate of dextrose infusion to maintain euglycaemia in the face of constant insulin infusion. Forearm insulin sensitivity is represented as forearm glucose uptake in μmol · min^{-1} · 100 ml^{-1}. Bars are S.E.M. Carbenoxolone increased whole-body insulin sensitivity without affecting peripheral insulin sensitivity, consistent with lowering intrahepatic cortisol concentrations as a result of inhibition of 11β-HSD1 reductase activity. (Results from [72].)

results in enhanced whole-body insulin sensitivity measured by the euglycaemic hyperinsulinaemic clamp technique, but does not alter peripheral insulin sensitivity measured by forearm glucose uptake [72] (Figure 5). This suggests that inhibition of hepatic 11β-HSD1 in man results in lower intrahepatic cortisol concentrations which in turn is associated with enhanced insulin-dependent down-regulation of hepatic glucose output.

4. In rats, oestrogen represses 11β-HSD1 expression in liver [73]. The direct effect of oestrogen, demonstrated in adrenalectomized rats, is to induce a rise in the gluconeogenic enzyme PEP-CK. However, in non-adrenalectomized rats with intact glucocorticoid secretion, oestrogen suppresses PEP-CK [74], consistent with enhanced insulin sensitivity due to lower re-activation of glucocorticoids in the liver by 11β-HSD1.

5. Transgenic deletion of the 11β-HSD1 gene in mice results in an inability to convert 11-dehydrocorticosterone into corticosterone (the equivalent of cortisone and cortisol, respectively, in man) and, despite elevated plasma corticosterone concentrations, is associated with impaired induction of hepatic gluconeogenic enzymes on starvation [75].

An additional level of complexity may influence the interaction between 11β-HSD1 and insulin action since insulin potently represses 11β-HSD1 expression [65,76]. It remains to be established whether 11β-HSD1 also influences insulin sensitivity in peripheral tissues such as skeletal muscle and fat. However, even if the effect is restricted to the liver, specific inhibitors of 11β-HSD1 might provide a useful therapeutic strategy to enhance insulin sensitivity in many different syndromes associated with insulin resistance.

EVIDENCE THAT GLUCOCORTICOID ACTIVITY IS INCREASED IN SUBJECTS WITH INSULIN RESISTANCE

From the above it is clear that excessive activity of glucocorticoids – whether by increased circulating levels of cortisol, increased glucocorticoid receptor sensitivity to cortisol or altered cortisol metabolism – is a plausible contributor to insulin resistance and could explain its association with hypertension, central obesity, dyslipidaemia and endothelial dysfunction. In addition, administration of glucocorticoids to rats *in utero* results in lower birthweight offspring which subsequently exhibit insulin resistance and hypertension [77,78], hence glucocorticoid excess provides a potential mechanism to explain the association of low birthweight with the metabolic syndrome [2,3].

A series of recent studies have examined the relationship between aspects of cortisol secretion and tissue action and cardiovascular risk factors.

Plasma cortisol concentrations and the hypothalamicñpituitaryñadrenal axis

In a large cross-sectional study we recently observed that plasma cortisol concentrations measured at 09.00 h are higher in adult men who were born with lower birthweight, and are associated with relative hypertension, insulin resistance, glucose intolerance and hypertriglyceridaemia [79] (Figure 6). Similar results have been obtained in other cohorts [80,81]. More recent data confirm that these men have evidence of chronic activation of the hypothalamic–pituitary–adrenal axis [81a]. The rate of cortisol secretion is also increased in young men with a familial predisposition to essential hypertension but not in men with a similar elevation of blood pressure whose parents had low blood pressure [82]. This suggests that increased cortisol secretion is an early, and perhaps inherited, feature of essential hypertension.

However, primary activation of the hypothalamic–pituitary–adrenal axis may not be responsible for increased cortisol secretion in all circumstances characterized by insulin resistance. The insulin resistance associated with obesity is in many ways distinct from insulin resistance in lean subjects, not least because it can usually be reversed by weight loss. Abnormalities of glucocorticoids are also different in lean and obese insulin-resistant subjects. The higher plasma cortisol observed in the studies described above appears to co-segregate with insulin resistance but not with obesity. Indeed, plasma cortisol is lower in obese subjects [83]. We have attributed this to enhanced metabolic clearance of cortisol by the enzyme 5α-reductase which is expressed in liver and fat [84]. The tendency to lower plasma cortisol may result in a compensatory increase in corticotrophin-releasing hormone, ACTH and cortisol

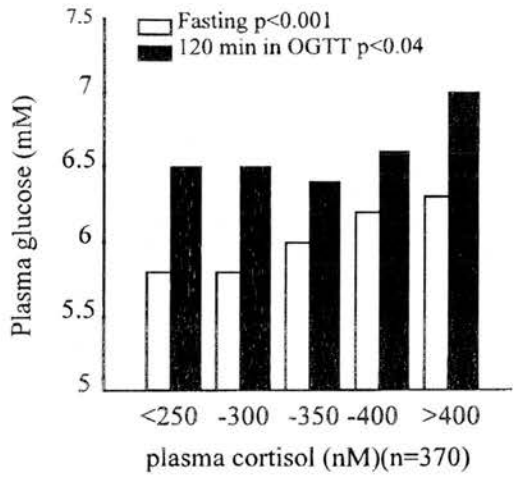

Figure 6 Plasma cortisol is elevated in subjects with glucose intolerance

In a cross-sectional study of 370 men aged 60ñ70 years in Hertfordshire, England, plasma cortisol was measured at 09.00 h after an overnight fast. Mean plasma glucose concentrations measured simultaneously and 2 h after a 75-g oral glucose tolerance test are shown for individuals in each of the quintiles of the distribution of plasma cortisol concentration. Higher plasma cortisol is associated with relative fasting hyperglycaemia and glucose intolerance. Similar associations were observed for insulin sensitivity, blood pressure and triacylglycerol levels. (Results from [79].)

secretion which may explain evidence of increased cortisol secretion in primary obesity [85,86]. The same effect has been invoked to explain the increased drive to adrenal steroidogenesis in the insulin-resistant polycystic ovarian syndrome [87], in which subjects are also usually obese. The mechanism of activation of the hypothalamic–pituitary–adrenal axis in non-obese subjects with insulin resistance remains uncertain, but may be distinct from that in obese subjects.

Glucocorticoid receptors

Assessment of glucocorticoid receptor sensitivity in man is difficult. Dexamethasone suppression tests assess central negative-feedback suppression of ACTH and cortisol secretion. Although the response to dexamethasone is variably reported as increased or impaired in obesity [88,89], it has not been reported to be abnormal in essential hypertension or lean insulin-resistant subjects.

An alternative test of peripheral glucocorticoid receptor sensitivity *in vivo* involves measuring the intensity of dermal blanching after topical administration of synthetic glucocorticoids. We found that this response is increased in patients with essential hypertension [90], in young adults with a familial predisposition to hypertension [82] and in men with relative glucose intolerance and insulin resistance [82]. Moreover, the dermal vasoconstrictor response to glucocorticoids is increased in healthy subjects who carry a polymorphism of the glucocorticoid receptor gene [91] which is more common in those with a familial predisposition to hypertension [92] and is associated with greater hyperinsulinaemia in obese subjects [93].

Glucocorticoid receptor function can also be measured *ex vivo* in leucocytes. Although these measurements do not relate to the polymorphism associated with increased dermal sensitivity [91], glucocorticoid receptors have a higher affinity for dexamethasone in leucocytes from subjects predisposed to hypertension [82]. On the other hand, in established essential hypertension, glucocorticoid receptor binding may be impaired [94].

These data suggest that glucocorticoid receptor sensitivity may be increased in the metabolic syndrome in peripheral tissues, but not in central tissues responsible for negative feedback. This inference has remarkable parallels in an animal model of the metabolic syndrome. In rats exposed to dexamethasone *in utero* who are born small and develop insulin resistance and hypertension as adults [77,78], glucocorticoid receptor expression is increased in their liver in association with up-regulation of the gluconeogenic enzyme PEP-CK [95]. However, central glucocorticoid receptor expression is down-regulated, explaining why these animals are relatively hypercorticosteronaemic [96]. It will be important to establish the mechanism for this apparent tissue-specific regulation of glucocorticoid receptor expression.

11β-Hydroxysteroid dehydrogenases

Cortisol metabolism by 11β-HSDs is also altered in subjects with insulin resistance, although these data are less consistent than the information concerning the glucocorticoid receptor. Insulin is a major inhibitor of 11β-HSD1 expression [65,76] so it would not be surprising if insulin resistance was associated with differences in the activity of this isoenzyme. In patients with essential hypertension, and in obese men, we and others have demonstrated a higher ratio of the metabolites of cortisol to those of cortisone and impaired conversion of labelled cortisol into cortisone [82,84,97,98]. However, in patients with the insulin resistance associated with polycystic ovarian syndrome, the reverse has been observed in some studies [99]. More sophisticated methods to dissect out the contribution of 11β-HSD1 and 11β-HSD2 in different tissues will be required to understand whether dysregulation of pre-receptor metabolism has a significant impact on activation of corticosteroid receptors in insulin-resistant subjects.

CONCLUSIONS

When Reaven and Hoffman described the associations between insulin resistance and other cardiovascular risk factors, they hypothesized that the insulin resistance might be the primary defect underlying the other features

[1]. This hypothesis is plausible if one allows the argument that only some aspects of insulin action are included in the resistance syndrome. Thus, resistance to the actions of insulin on glucose metabolism leads to relative hyperglycaemia with compensatory hyperinsulinaemia; but if the effects of insulin are maintained – for example with respect to tissue growth, renal salt excretion [100] and adrenal androgen steroidogenesis – then the hyperinsulinaemia may promote atherogenesis, hypertension and polycystic ovarian syndrome respectively.

This hypothesis has become more complicated with time and has proved difficult to test. For example, the association between insulin resistance and endothelial dysfunction can only be explained by a primary resistance to insulin if both glucose metabolism and the endothelium are resistant to insulin action. Similarly, the association between low birthweight and subsequent insulin resistance could be explained if the insulin resistance includes insulin-mediated growth and development *in utero*. However, in support of the original hypothesis, there are rare examples of mutations resulting in dissociation of the growth-promoting and glucose-regulating actions of insulin [101]. Moreover, although homozygous mutations of the insulin receptor cause a clinical syndrome including lipoatrophy [7], transgenic animals with global insulin resistance due to heterozygous multiple mutations of the insulin receptor signalling pathway do turn out to be obese with other features of the metabolic syndrome [102]. Finally, newer drugs which enhance insulin sensitivity and improve glucose tolerance [35] also improve other aspects of the metabolic syndrome, including reproductive function [6] and, in animals at least, blood pressure.

In the absence of a clear understanding of the cause of insulin resistance in most subjects, and given that uncertainty remains about whether insulin resistance is an important primary mediator in the metabolic syndrome, there is scope to consider alternative hypotheses to explain these associations. For example, both insulin resistance and other cardiovascular risk factors may result from a common primary abnormality. This review illustrates the plausibility of a hypothesis that enhanced activity of cortisol contributes to insulin resistance, and that manipulation of cortisol action provides a novel therapeutic target to enhance insulin sensitivity. Clearly, further work is required to address whether alterations in cortisol secretion and sensitivity are causes or consequences of insulin resistance, to understand the molecular mechanisms for these alterations, and to characterize in more detail the targets for glucocorticoid effects on insulin sensitivity.

Cortisol and insulin were both discovered in the same era, and in an early phase of endocrine research. Both have transformed clinical practice in this century. Arguably, insulin has stolen the limelight in recent years, and cortisol has been eclipsed by research into numerous 'novel' cardiovascular hormones. However, as illustrated in this review, you *can* teach an old dog new tricks.

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Cortisol Metabolism in Healthy Young Adults: Sexual Dimorphism in Activities of A-Ring Reductases, but not 11β-Hydroxysteroid Dehydrogenases*

MARTIJN J. J. FINKEN, ROBERT C. ANDREWS, RUTH ANDREW, AND BRIAN R. WALKER

University of Edinburgh, Endocrinology Unit, Department of Medical Sciences, Western General Hospital, Edinburgh, United Kingdom EH4 2XU

ABSTRACT

Cortisol is metabolized irreversibly by A-ring reductases (5α- and 5β-reductases) and reversibly (to cortisone) by 11β-hydroxysteroid dehydrogenases (11βHSDs). In rats, estradiol down-regulates 11βHSD1 expression. In humans, ratios of urinary cortisol/cortisone metabolites differ in men and women. In this study, urinary cortisol metabolites and hepatic 11βHSD1 activity were measured in healthy young men and women at different phases of the menstrual cycle.

Ten men and 10 women with regular menstrual cycles collected a 24-h urine sample, took 250 μg oral dexamethasone at 2300 h, took 25 mg oral cortisone at 0900 h (after fasting), and had blood sampled for plasma cortisol estimation over the subsequent 150 min. Women repeated the tests in random order in menstrual, follicular, and luteal phases.

Women excreted disproportionately less A-ring-reduced metabolites of cortisol [median 5α-tetrahydrocortisol, 1811 (interquartile range, 1391–2300) μg/day in menstrual phase *vs.* 2723 (interquartile range, 2454–3154) in men ($P = 0.01$); 5β-tetrahydrocortisol, 1600 (interquartile range, 1419–1968) *vs.* 2197 (interquartile range, 1748–2995; $P = 0.03$)] but similar amounts of cortisol, cortisone, and tetrahydrocortisone. Analogous differences were observed in urinary excretion of androgen metabolites. Conversion of cortisone to cortisol on hepatic first pass metabolism was not different (peak plasma cortisol, 733 ± 60 nmol/L in women *vs.* 684 ± 53 nmol/L in men; mean ± SEM; $P = 0.55$). There were no differences in cortisol or androgen metabolism between phases of the menstrual cycle.

We conclude that sexual dimorphism in cortisol metabolite excretion is attributable to less A-ring reduction of cortisol in women, rather than less reactivation of cortisone to cortisol by 11βHSD1. This difference is not influenced acutely by gonadal steroids. 11βHSD1 has been suggested to modulate insulin sensitivity and body fat distribution, but caution must be exercised in extrapolating inferences about its regulation from rodents to man. A-Ring reductases may have an equally important influence on metabolic clearance of cortisol and intracellular cortisol concentrations. (*J Clin Endocrinol Metab* **84:** 3316–3321, 1999)

CORTISOL IS metabolized by several enzymes (Fig. 1), including irreversible inactivation by A-ring reductases (5α- and 5β-reductases) and reversible interconversion to inactive cortisone. Interconversion with cortisone is catalyzed by 11β-hydroxysteroid dehydrogenases (11βHSDs), which are now recognized to play a crucial role in modulating activation of corticosteroid receptors. 11βHSD type 2 (1, 2) inactivates cortisol in the distal nephron, thereby protecting mineralocorticoid receptors from inappropriate activation by cortisol. Congenital or acquired defects in 11βHSD2 result in cortisol-dependent mineralocorticoid excess (3–5). More recently, the role of 11βHSD type 1 (6) has been defined. This enzyme reactivates cortisone in many sites, including liver and adipose tissue (7–9), where it appears to maintain adequate exposure of glucocorticoid receptors to cortisol (10–12). Defects in 11βHSD1 result in enhanced sensitivity to insulin. Increased activity of 11βHSD1 has been postulated to be important in insulin resistance syndromes, particularly obesity (9, 13, 14).

A number of studies in rodents, many of which preceded the cloning of distinct 11βHSD1 and 11βHSD2 isozymes, have examined the regulation of these enzymes. In brief, 11βHSD2 is constitutive and appears to present an effective barrier to glucocorticoid access to mineralocorticoid receptors under all conditions. By contrast, 11βHSD1 is regulated by glucocorticoids (15), thyroid hormones (16), insulin (15), GH (17), cytokines (18), and gonadal steroids (17, 19–21). Regulation of 11βHSD1 by gonadal steroids is of particular interest, because sex-specific differences in enzyme activity could contribute to differences in body fat distribution and susceptibility to cardiovascular risk factors associated with insulin resistance.

In rats, 11βHSD1 expression and activity in liver are markedly lower in females than in males (21). Estradiol administration to gonadectomized rats potently represses 11βHSD1 expression, an effect that depends at least in part on changes in the pattern of GH secretion (17). Evidence of whether estrogen regulates 11βHSD1 in humans is surprisingly limited. In premenopausal healthy women, the ratio of urinary metabolites of cortisol to cortisone has been reported to be lower than that in men (22), but the characteristics of participants in that study were not described in detail. The same trend was observed in hypopituitary patients (23), but clearly there are potential confounding effects of hormonal

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Address all correspondence and requests for reprints to: Dr. Brian R. Walker, University of Edinburgh, Endocrinology Unit, Department of Medical Sciences, Western General Hospital, Edinburgh, United Kingdom EH4 2XU. E-mail: b.walker@ed.ac.uk.

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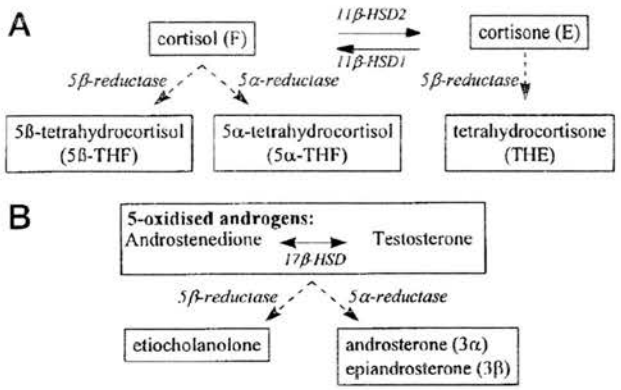

FIG. 1. Principal metabolites of cortisol (A) and androgens (B) measured in urine by gas chromatography and mass spectrometry. *Dotted arrows* indicate that more than one step is involved.

replacement therapies in this group. In healthy postmenopausal women, the ratio of cortisol/cortisone metabolites was higher than that in men and was not influenced by estrogen replacement therapy (14).

In this study, we sought to clarify whether sex-specific difference in cortisol metabolism are observed in healthy young adults and to establish whether changes in urinary cortisol/cortisone metabolites could be attributed to differences in hepatic conversion of cortisone to cortisol by 11βHSD1. In addition, we sought differences in these indexes of cortisol metabolism in different phases of the menstrual cycle.

Experimental Subjects

Lothian Research ethics committee approval and written informed consent were obtained. All participants were white Caucasians. Ten healthy men and 10 healthy women with regular endogenous menstrual cycles (between 24–34 days) were recruited by advertisement. Their characteristics are shown in Table 1. Groups were matched for age and body mass index. Inclusion criteria were: age, 20–45 yr; blood pressure, less than 160/90 mm Hg; no regular medication; no use of glucocorticoid therapy by any route during the previous 3 months; no psychiatric illness in the previous 3 months; and no abnormality of renal, thyroid, or liver function on biochemical screening.

Materials and Methods

Clinical protocol

Men were studied on one occasion. Women were studied on three occasions, in random order, during menstrual (2–5 days after starting menstruation), follicular (19–16 days before the next expected menstruation), or luteal (9–5 days before next menstruation) phases. All studies were completed in the winter months (December to March), and men and women were studied in parallel to avoid confounding effects of seasonal changes in steroid metabolite excretion (24).

On each occasion, subjects collected a 24-h urine sample, took 250 μg oral dexamethasone at 2300 h, and attended next day at 0830 h after an overnight fast. A venous cannula was inserted, and blood was withdrawn after 25 min for cortisol and, in women, estradiol and progesterone determinations. After 30 min, 25 mg oral cortisone acetate was administered, and blood was sampled during the next 150 min for plasma cortisol determination. The dose of dexamethasone was selected to lower baseline plasma cortisol so that a rise could be readily detected after cortisone administration without giving so much that dexamethasone metabolites might interfere with cortisone metabolism (25).

Laboratory measurements

Cortisol and its metabolites in urine were measured by gas chromatography and electron impact mass spectrometry after Sep-Pak C_{18} extraction, hydrolysis with β-glucuronidase, and formation of methoxime-trimethylsilyl derivatives as previously described (26). Epi-cortisol and epi-tetrahydrocortisol were used as internal standards, which were added to samples before extraction. Peaks of interest were quantified by the ratio of (area under the peak)/(area under neighboring internal standard peak), rather than the ratio of peak height against a line extrapolated from bracketed internal standards as used by many other groups. Ratios were compared against standard curves for each steroid included in every assay batch. The average intraassay precision for all steroids measured was less than 20% (n = 16 assays). In each assay batch, water samples were included containing standard steroids and average accuracy for all steroids varied from −4.4% to +5.5% (n = 16). Principal urinary androgen metabolites were measured using the same method, except that 5α-androstan-3α,17α-diol was used as an internal standard. Pathways of cortisol and androgen metabolism leading to these metabolites are illustrated in Fig. 1. The ratio between 5β-reduced and 5α-reduced metabolites of cortisol and androgens was closely correlated ($r = 0.80$; $P < 0.001$).

Cortisol, estradiol, and progesterone were measured in plasma by commercial RIAs.

TABLE 1. Characteristics of participants

	Males (n = 10)	Females (n = 10)	*P* value (by Student's *t* test)
Age (yr)	27.8 ± 1.5 (22–35)	28.6 ± 1.7 (20–40)	0.61
Ht (m)	1.80 ± 0.02 (1.73–1.91)	1.63 ± 0.02 (1.55–1.71)	<0.0001
Wt (kg)	77.4 ± 3.7 (60.3–96.7)	65.9 ± 3.8 (47.8–94.5)	0.04
Body mass index (kg/m²)	23.9 ± 1.0 (18.6–27.3)	24.9 ± 1.6 (18.4–34.3)	0.63
Waist circumference (cm)	87 ± 2 (71–99)	76 ± 4 (61–97)	0.02
Hip circumference (cm)	100 ± 2 (91–113)	97 ± 3 (80–110)	0.34
Waist/hip ratio	0.87 ± 0.01 (0.78–0.91)	0.78 ± 0.02 (0.66–0.92)	0.002

Data are the mean ± SEM (range). Values for females were recorded during the menstrual phase.

Statistics

Results are presented as the mean ± SEM for normally distributed variables; groups were compared using unpaired Student's *t* tests or repeated measures ANOVA. Many of the urinary cortisol metabolites had skewed distributions, necessitating nonparametric analyses; these are presented as median (interquartile range). Data from men and women in the menstrual phase were compared by Mann-Whitney U tests; data from different phases of the menstrual cycle were compared by Friedman ANOVA. Data for men and women in luteal and follicular phases were not compared to avoid multiple statistical testing.

Results

Comparison between men and women

Men and women were well matched for age and body mass index, but men were taller and heavier, with android distribution of body fat (Table 1). Absolute excretion rates of urinary cortisol metabolites tended to be higher in men (Table 2). This was attributable principally to lower excretion of 5α-reduced and 5β-reduced metabolites of cortisol in women. Excretions of cortisol, cortisone, and tetrahydrocortisone were not different. Excretion of androgen metabolites also tended to be higher in men, accounted for by a trend toward lower excretion of 5α-reduced metabolites in women.

Table 2 shows ratios of metabolites reflecting activities of

TABLE 2. Twenty-four-hour urinary cortisol and androgen metabolite excretion

	Males	Females			*P* males vs. menstrual females (Mann-Whitney U test)	*P* between menstrual phases (Friedman ANOVA)
		Menstrual	Follicular	Luteal		
Cortisol	119 [85–139]	150 [84–204]	163 [115–178]	117 [71–256]	0.50	0.41
Cortisone	135 [115–150]	123 [85–145]	116 [111–146]	133 [122–195]	0.36	0.90
5α-THF	2723 [2454–3154]	1811 [1391–2300]	1950 [1698–2324]	1943 [1765–2245]	0.01	0.27
5β-THF	2197 [1748–2995]	1600 [1419–1968]	1770 [1412–1965]	2007 [1434–2259]	0.03	0.74
THE	2393 [2082–2895]	1927 [1678–2860]	2238 [1789–2735]	1999 [1525–2515]	0.55	0.67
Sum of cortisol metabolites[a]	11879 [9963–20644]	10140 [7360–11430]	10321 [8811–14718]	12568 [7068–19285]	0.10	0.41
Cortisol/cortisone	0.94 [0.73–1.04]	1.27 [0.94–1.70]	1.16 [0.91–1.74]	0.92 [0.73–1.37]	0.06	0.49
5β/5α-THF	0.76 [0.66–1.16]	0.92 [0.69–1.30]	0.86 [0.70–1.00]	0.98 [0.79–1.07]	0.45	0.50
THFs/THE	1.99 [1.70–2.62]	1.90 [1.38–2.45]	1.78 [1.34–2.01]	2.06 [1.39–2.63]	0.50	0.67
5α-THF/cortisol	23.4 [20.5–27.7]	18.8 [5.9–24.1]	12.2 [10.4–20.2]	17.1 [8.8–26.7]	0.07	0.74
5β-THF/cortisol	16.0 [14.6–30.4]	12.6 [9.0–16.8]	11.7 [7.4–15.3]	14.2 [9.2–18.9]	0.03	0.50
THE/cortisone	18.3 [14.5–22.8]	19.4 [15.1–20.9]	18.5 [15.8–19.9]	13.5 [12.5–18.1]	0.55	0.27
5-Oxidized androgens (androstenedione + testosterone)	186 [106–209]	254 [145–316]	170 [130–245]	255 [101–432]	0.13	0.12
5α-Reduced androgens (androsterone + epiandrosterone)	3775 [3213–4951]	3294 [2356–3962]	3099 [1819–4330]	3069 [2277–3709]	0.15	0.50
5β-Reduced androgens (etiocholanolone)	2191 [1076–2566]	1881 [1384–2492]	1628 [1234–2571]	2122 [1209–2651]	0.88	0.74
Sum of androgen metabolites[b]	6267 [4395–7435]	5249 [4484–6916]	4808 [3080–7146]	5816 [3632–6835]	0.60	0.74
5β/5α-Reduced androgens	0.53 [0.35–0.68]	0.68 [0.55–0.98]	0.59 [0.47–0.85]	0.64 [0.56–0.77]	0.11	0.27
5α-Reduced/5-oxidized androgens	25.9 [20.1–32.3]	14.2 [11.2–16.6]	19.0 [14.9–24.3]	15.6 [9.4–27.3]	0.02	0.08
5β-Reduced/5-oxidized androgens	10.8 [9.5–17.5]	9.0 [7.8–11.0]	9.7 [7.0–14.6]	8.7 [5.8–20.4]	0.23	0.50

Data are medians [inter-quartile range], expressed as micrograms per 24 h.

[a] Sum of urinary cortisol metabolites = 5α-THF + 5β-THF + THE + cortols + cortolones.

[b] Sum of urinary androgen metabolites = dehydroepiandrosterone + androstenedione + testosterone + androsterone + epiandrosterone + etiocholanolone.

TABLE 3. Plasma cortisol

	Males	Females			*P* males *vs.* menstrual females (Student's *t* test)	*P* between menstrual phases (repeated measures ANOVA)
		Menstrual	Follicular	Luteal		
Fasting baseline plasma cortisol (nmol/L)[a,b]	323 ± 74	268 ± 37	272 ± 43	270 ± 54	0.52	0.99
Peak plasma cortisol (nmol/L)[a]	684 ± 53	733 ± 60	800 ± 62	792 ± 58	0.55	0.49
Time to peak (min)	75 ± 8	111 ± 9	99 ± 13	86 ± 7	0.01	0.27
Area under curve[c]	221 ± 162	343 ± 139	305 ± 157	352 ± 156	0.10	0.37

Data are the mean ± SEM

[a] Subjects received 250 μg oral dexamethasone at 2300 h the previous evening and 25 mg oral cortisone acetate on the morning of the test.

[b] Baseline plasma cortisol was calculated as the arithmetic mean of measurements at 5 and 0 minutes before cortisone administration.

[c] Calculated by the trapezoidal rule as the average increment above baseline plasma cortisol from 15–150 min.

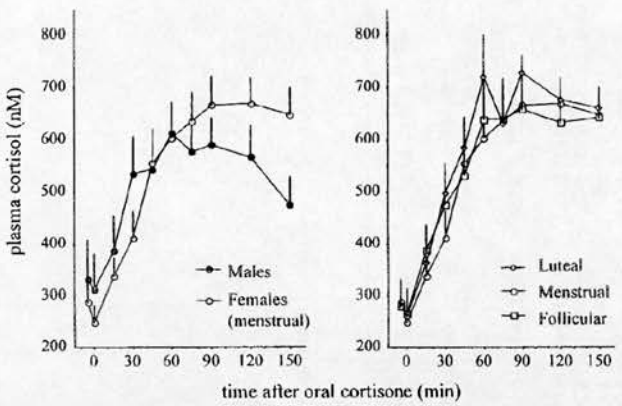

FIG. 2. Hepatic 11βHSD1 activity. Plasma cortisol was measured after overnight dexamethasone suppression (250 μg, orally, at 2300 h) and oral administration of 25 mg cortisone acetate at 0900 h. Data are the mean ± SEM for males (n = 10), and females (n = 10) in menstrual, follicular, and luteal phases of the endogenous menstrual cycle. Comparisons are given in Table 3.

different enzymes. 11βHSD activities are conventionally inferred from relative excretion of metabolites of cortisol and cortisone [cortisol/cortisone ratio reflecting principally renal 11βHSD2 activity (26, 27) and ratios of tetrahydrocortisols/tetrahydrocortisone reflecting the balance between 11βHSD1 and 11βHSD2 activities]; these ratios did not differ between men and women, although there was a trend for a lower cortisol/cortisone ratio in men. A-Ring 5α- and 5β-reductase activities can be inferred relative to each other by the ratio of 5β-tetrahydrocortisol/5α-tetrahydrocortisol and by 5β-/5α-reduced androgen metabolites, which did not differ between men and women. Provided that urinary cortisol and cortisone are not different (28), 5β-reductase activity can also be inferred from the ratios of 5β-tetrahydrocortisol/cortisol, tetrahydrocortisone/cortisone (29), and 5β-reduced/5-oxidized androgen metabolites; in women this activity was less for cortisol but not for cortisone or androgens. 5α-Reductase activity can be inferred from the ratio of 5α-tetrahydrocortisol/cortisol and 5α-reduced/5-oxidized androgens, which was lower in women.

Plasma cortisol after overnight dexamethasone suppression was not different between men and women (Table 3). The rise in plasma cortisol after oral cortisone administration is shown in Fig. 2. The rate of rise, maximum plasma cortisol, and area under the curves did not differ between men and women. However, the peak cortisol occurred earlier in men than women, apparently because cortisol was cleared from plasma more quickly in men.

Comparison between phases of menstrual cycle in women

Accuracy of timing of phases of the menstrual cycle was confirmed by measurements of plasma estradiol and progesterone (data not shown). For all women, estradiol was higher in the follicular than in the menstrual phase, and progesterone was only detectable in the luteal phase.

There were no differences in urinary cortisol or androgen metabolite excretion (Table 2) or conversion of oral cortisone to plasma cortisol (Fig. 2) in different phases of the menstrual cycle.

Discussion

This study is consistent with previous observations that urinary excretion of the A-ring reduced metabolites of cortisol is lower in premenopausal women than in men (22), and that this is out of proportion to the excretion of other metabolites of cortisol in women. The disproportionality of these differences excludes technical confounders, such as incomplete urine collection. The absolute excretion rates for some cortisol metabolites, particularly 5α-tetrahydrocortisol, are somewhat higher in this group than those reported by others in healthy volunteers (3, 27, 30, 31). This may reflect differences between groups of subjects or methodological differences, for example in the choice of internal standards. In addition, this study confirms previous reports that urinary androgen excretion is only marginally higher in men than in women (30), reflecting the fact that most urinary androgen metabolites are derived from adrenal androgens.

Previously, differences in cortisol metabolite excretion between men and women have been attributed to alterations in 11β-hydroxysteroid dehydrogenases. Whether this reflects enhanced inactivation of cortisol to cortisone by 11βHSD2 or impaired reactivation of cortisone to cortisol by 11βHSD1 in women had not been tested. However, it was assumed that lesser 11βHSD1 activity in women was responsible on the basis of studies in rats suggesting that only 11βHSD1 is regulated by other hormones, including down-regulation by estrogen (17, 20, 21).

In the present study, ratios of urinary cortisol/cortisone (26, 27) suggest that conversion of cortisol to cortisone by renal 11βHSD2 activity is lower, rather than higher, in women than men. Therefore, differences in 11βHSD2 could not explain lower ratios of cortisol/cortisone metabolites; this is consistent with the hypothesis that these differences reflect sexual dimorphism in 11βHSD1 activity. However, we also made a more specific assessment of hepatic 11βHSD1 activity by measuring the conversion of cortisone adminis-

tered orally into cortisol in the peripheral circulation. The rate of appearance of cortisol is lower when 11βHSD1 is inhibited, *e.g.* by carbenoxolone (32), but is not influenced by 11βHSD2 activity (33). The lack of sexual dimorphism in the rate of appearance of cortisol in the present study suggests that 11βHSD1 activity is not different in men and women. Moreover, in marked contrast with dramatic changes over a similar time course in rats (17, 21), changes in estrogen levels in women during the menstrual cycle were not associated with alterations in any index of 11βHSD1 activity.

The present data suggest an alternative explanation for the disproportionately low excretion of tetrahydrocortisols in women. As previously described in postmenopausal (14) and hypopituitary (23) women, the excretion of cortisol and cortisone is similar or even increased in women compared with that in men; the differences are observed only in the A-ring reduced metabolites. Thus, the ratios of urinary metabolites suggest that rates of A-ring reduction of cortisol are lower in women than in men. This is substantiated by examining A-ring reduction of androgen metabolites. Lower A-ring reduction could also explain why cortisol is cleared less rapidly from plasma in women than in men after an oral dose of cortisone. Unlike postmenopausal women (14), this difference does not affect the 5β-reduction of cortisone and could therefore account both for the lower ratio of tetrahydrocortisols/tetrahydrocortisone observed in young women (22), but not postmenopausal women (14), and for the trend toward higher urinary cortisol/cortisone in women in this study.

It is not clear why A-ring reduction of cortisol should differ between men and women. The principal enzymes involved are 5β-reductase (34) and 5α-reductase types 1 and 2 (35). 5β-Reductase is expressed in liver and is involved in the metabolism of bile acids. Although there is some evidence that affinities for cortisol and cortisone/androgens can be separated by semipurification and subcellular fractionation *in vitro* (36, 37), there is no evidence that there is more than one 5β-reductase active *in vivo* in man (38). 5β-Reductase activity is lower in female than in male rat livers (39), but it is up-regulated by estrogen (40). 5α-Reductase type 1, the principal isozyme in human liver and fat (41), is usually thought to be constitutive and not regulated hormonally (42, 43), but there is some evidence that this isozyme is down-regulated by androgens (44, 45) more so than by estrogen (46), so that its activity is higher in female liver (47) and adrenal (48). 5α-Reductase type 2 is expressed mainly in the prostate and is up-regulated by androgens (35). These observations from animals predict that activities of 5β-reductase may be lower, and hepatic 5α-reductase may be higher, rather than decreased, in women. Moreover, the lack of acute effect of changes in gonadal steroids on urinary cortisol metabolite excretion in the current study suggests that the explanation for sexual dimorphism in A-ring reductases does not relate to acute gonadal steroid regulation in humans. An alternative explanation relates to the relative mass of tissues expressing A-ring reductases in men and women. It is not clear whether the prostate contributes substantially to A-ring reduction of cortisol, but, interestingly, finasteride, a relatively specific inhibitor of 5α-reductase type 2, does alter the relative excretion of cortisol metabolites in men (49). The quantity and distribution of body fat may also be important, as 5α-reductase type 1 is expressed in adipocytes and is more active in peripheral sc than central visceral fat in culture (41), although the contribution of adipose 5α-reductase activity to cortisol clearance may be small. Previous studies suggest that increased visceral fat in men may be associated with greater 5α-reductase activity (14). However, the current study is too small, and subjects within it too similar, to explore whether differences in body fat distribution might explain sexual dimorphism in cortisol metabolite excretion.

We have previously reported cortisol metabolite profiles in older subjects, in whom we found relationships between greater central/visceral obesity and enhanced activity of 5α-reductase, but not 5β-reductase (14). The pattern of differences between older men and women contrasts with the results in young subjects studied here and previously (22). Older women had higher 5α-reductase, but not 5β-reductase, activity and higher ratios of metabolites of cortisol to those of cortisone in urine compared with men. Comparing values in Table 2 with values in the older population measured by the same method (14), it appears that there is little difference between postmenopausal and premenopausal women, but that the major differences are between younger and older men. Thus, aging in men may be associated with falling activities of 5α-reductase and 11βHSD1.

Whatever the reason for sexual dimorphism of A-ring reduction of cortisol and its change with age, this observation has important implications for physiological glucocorticoid action and for interpretation of apparent pathological disruption of cortisol metabolism. Lesser A-ring reduction of cortisol in women predicts a lower MCR of cortisol, which, in turn, predicts greater feedback suppression of the hypothalamic-pituitary-adrenal axis. If A-ring reduction is increased, as in obesity (14) and probably also in polycystic ovarian syndrome (50, 51), increased ACTH drive to the adrenal cortex may contribute to excessive androgen secretion. Conversely, a decline in A-ring reduction, as may occur with normal aging in men, may contribute to the fall in adrenal androgen excretion with age (52). In addition, the extent of A-ring reduction in specific organs, including adipose tissue and liver, will influence local concentrations of cortisol independently of circulating glucocorticoid concentrations. It remains to be established whether this has a potent influence on corticosteroid receptor activation, but it may contribute to the sexual dimorphism of body fat distribution.

Arguably the most important implications of this study are that care should be exercised in extrapolating to humans from studies of regulation of 11βHSD1 in rodents, and that ratios of tetrahydrometabolites of cortisol and cortisone should be interpreted cautiously if they are not accompanied by measurements of cortisol and cortisone (26, 27). The latter has not been measured in some other studies comparing men and women (22) or in studies of polycystic ovarian syndrome (50, 51) or essential hypertension (53, 54). Arguably for this reason the inferences concerning 11βHSD activities may have been overemphasized, and the potential importance of disturbances in A-ring reduction of cortisol may have been overlooked.

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